2017

Evaluation of methods for generating Senecavirus A virus-like particles utilizing the baculovirus expression vector system

Jennifer Lynn English
Iowa State University

Evaluation of methods for generating Senecavirus A virus-like particles utilizing the baculovirus expression vector system

by

Jennifer Lynn English

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Microbiology

Program of Study Committee:
James Roth, Co-major Professor
Eric Vaughn, Co-major Professor
Kenneth Stalder

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2018

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

AcNPV	*Autographa californica* Nuclear Polyhedrosis Virus
ADG	Average Daily Gain
BEVS	Baculovirus Expression Vector System
BV	Budded Virus
CVA	Coxsackievirus A
DIVA	Differentiation of Infected from Vaccinated Animals
DNA	Deoxyribonucleic Acid
EMCV	Encephalomyocarditis Virus
EM	Electron Microscopy
ETNL	Epidemic Transient Neonatal Losses
EV71	Enterovirus 71
FMD	Foot-and-Mouth Disease
FMDV	Foot-and-Mouth Disease Virus
HAV	Hepatitis A Virus
IRES	Internal Ribosomal Entry Site
ISU VDL	Iowa State University Veterinary Diagnostic Lab
kDa	Kilodalton
NADC	National Animal Disease Center
NT	Nucleotides
ODV	Occlusion-derived Viruses
OIE	Office International des Epizooties
ORF	Open Reading Frame

PCR	Polymerase Chain Reaction
PTM	Post-translational Modifications
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SIVD	Swine Idiopathic Vesicular Disease
SVD	Swine Vesicular Disease
SVDV	Swine Vesicular Disease Virus
SVV	Seneca Valley Virus
TBS	Tris-buffered Saline
TEM	Transmission Electron Microscopy
USDA	United States Department of Agriculture
UTR	Untranslated Region
VE	Vesicular Exanthema
VEV	Vesicular Exanthema Virus
VS	Vesicular Stomatitis
VSV	Vesicular Stomatitis Virus
VLP	Virus-like Particle
VPg	Viral Protein, genome linked

ACKNOWLEDGMENTS

I would like to thank my Co-major professors Dr. Eric Vaughn and Dr. James Roth for their guidance and expertise throughout the scientific and writing process of the work contained in this thesis. Dr. Vaughn has shown complete support from beginning to end, and I appreciate his trust and confidence in my abilities for completing this milestone in my career. Dr. Roth agreed to be a Co-major professor as I enrolled as an untraditional graduate student. He has put in much effort in assisting with the requirements of the graduate program with little in return; if it was not for him, I would not have been able to start and complete this degree. I, also, owe a huge thank you to Dr. Ken Stalder for not only serving as a committee member for my Master's, but for being the first professor when I was an undergraduate to realize my potential as a graduate student and encouraging me to further my education.

I would like to express my gratitude to all of those at BIAH who have worked with me on many different levels to reach this accomplishment. Luis Hernandez is an outstanding mentor and is always challenging me to improve as a scientist. I could not have done this without his guidance and support. Scott Ackerman was a great team player assisting me with lab work when I had to run off to class. Arun Iyer spent many hours of his personal time providing constructive revisions and advice during the review process of my thesis, as well as, continually checking in on my progress and providing encouragement. I am thankful to work with such a supportive group of colleagues within a company that believes in personal development.

Lastly, I would like to thank my family and friends for their love and support on the home front. They have downloaded countless articles I needed for my thesis, provided me

with meals, dog sat my beloved Bella and Daisy and have been understanding in my lack of presence, especially in these last few months. I am very grateful to have been given such a great opportunity for my personal development and professional career.

ABSTRACT

A recombinant subunit vaccine against Seneca Valley virus (SVV) would be valuable to the swine industry. Recent SVV outbreaks have raised concerns with national biosecurity. This is due to the potential of foreign vesicular animal diseases entering the United States undetected because of confounding clinical signs of SVV. Research with the baculovirus expression vector system (BEVS) has produced virus-like particles (VLP) that elicited neutralizing antibodies and protected against challenge for many *Picornaviridae* viruses. Building on this previous research, attempts of SVV VLP assembly for a vaccine candidate were performed using the BEVS.

All baculovirus constructs were designed to encode the SVV P1 region, 2A protease, portions of the 2B and 3B genes and 3C protease. Recombinant SVV proteins VP1, VP2 and VP3 were expressed in several baculovirus construct iterations. Peptide specific antibodies detected each protein by means of Western blot analysis. Sucrose gradient fractionation and electron microscopy (EM) were performed to verify VLP formation. However, VLP production was not confirmed by either method.

Several factors influence the assembly of SVV VLPs. One outcome providing insight of the complete cleavage of the recombinant capsid proteins is the identification of a 55kDa protein band. This band was detected in the α-SVV VP1 and α-SVV VP3 Western blots. Also, the 3C protease and its impact in the BEVS need further investigation to determine its role in complete cleavage of the recombinant capsid proteins. Lastly, there were indications the recombinant capsid proteins were aggregating instead of folding properly into VLPs. The gaps in information noted here support the need for further research into capsid formation in *Picornaviridae* viruses to improve VLP assembly using the BEVS.

CHAPTER 1: INTRODUCTION

Background

Seneca Valley virus (SVV) is the common virus name for the *Senecavirus A* species which is the only species within the *Senecavirus* genus in the *Picornaviridae* family[1]. The *Picornaviridae* family also includes the foreign animal disease viruses, Foot and Mouth Disease Virus (FMDV) and Swine Vesicular Disease Virus (SVDV). Pigs infected with SVV, FMDV or SVDV present similar clinical signs of fluid filled vesicles on the snout and coronary bands of the hoof. Seneca Valley virus has circulated in US herds for many years, but recent outbreaks with clinical signs have raised concerns with biosecurity. Inadvertently allowing FMDV or SVDV to enter US swine herds due to under reporting of SVV would be devastating to the swine industry. Providing a vaccine for SVV would reduce the prevalence of vesicle presentation in swine in turn decreasing the risk of failing to comply in the reporting of vesical clinical signs. This would support rapid identification of harmful foreign diseases of swine exhibiting similar clinical signs.

Objectives

The objectives of this research were to: 1. Design and engineer baculovirus constructs encoding SVV capsid proteins. 2. Evaluate the expression of the SVV recombinant capsid proteins in baculovirus-infected insect cells. 3. Assess the SVV recombinant capsid proteins from baculovirus-infected insect cells for VLP formation by sucrose gradient fractionation and electron microscopy (EM) imaging.

Thesis Organization

This thesis consists of an introduction, literature review, one original research chapter and a conclusion. Chapter 1 serves as the introduction followed by the literature review in Chapter 2. Topics included in the Chapter 2 literature review begin with the overall characteristics of the *Picornaviridae* family then discuss in more detail the genome, capsid formation, disease, history and current status of Seneca Valley virus. The literature review also covers a general overview of vaccine approaches in the animal health industry, VLP vaccines, baculovirus biology, harnessing the baculovirus platform technology for protein expression and successful application of the BEVS for VLP assembly of *Picornaviridae* proteins. Chapter 3 contains original research for designing and constructing baculovirus constructs for expression and assembly of SVV VLPs. Chapter 4 consists of a conclusion summarizing the material presented. The author's role in this research included the assembly of baculovirus constructs, purposeful genetic manipulation of gene fragments, PCR screening, transfection and harvest of baculovirus infected insect cells, evaluation of expressed proteins by SDS-PAGE and Western blotting, analysis and interpretation of results and manuscript writing.

CHAPTER 2: LITERATURE REVIEW

Characteristics of the Picornaviridae family

The *Picornaviridae* family currently consists of 35 genera including *Senecavirus* [2]. Picorna is derived from *pico* meaning small and RNA referring to the RNA genome that all family members possess [3]. Viruses in this family have a viral capsid that surrounds the positive-sense, single-stranded RNA genome that is approximately 7500-8500 nucleotides (nt) [4,5]. A single open reading frame (ORF) encodes for structural proteins located at the 5' end and proteins responsible for protein processing and viral replication encoded at the 3' end [6]. The ORF is translated into a single polyprotein that is cleaved into the individual functional proteins [4,5]. Covalently attached at the 5' N-terminal end of the genome is a viral-encoded protein VPg that is responsible for initiation of RNA synthesis followed by a 5' untranslated region (UTR) [4,5]. In this UTR, an internal ribosome entry site (IRES) is present which allows RNA translation to occur in a cap-independent manner [5].

After the 5' UTR, many picornavirus genera have a standard L-4-3-4 protein layout consisting of a Leader protein-P1 region with 4 polypeptides-P2 region with 3 polypeptides-P3 region with 4 polypeptides (Fig. 1) [2,5-7]. The P1 region encodes for the icosahedral viral capsid that contains 60 copies of VP1 and VP3 subunits, 58-59 copies of VP2 and VP4 subunits and one or two copies of VP0, a precursor protein of VP2 and VP4 [2,4,8,9]. However, there are several picornavirus genera where VP0 remains uncleaved in the final capsid structure [2]. The P2 and P3 regions encode nonstructural proteins 2A, 2B, 2C, and 3A-3D for protein processing and viral replication [2]. The 2A, 3C and 3C-precursor proteins are responsible for cleaving the single polyprotein resulting in the functional subunit proteins.

The 2A protease cleaves the structural proteins from the nonstructural and 3C and 3C- precursors complete the other cleavages. The orthologous proteins 1B, 1C, 1D, 2C, 3C and 3D are conserved in all picornaviruses while 1A, 2A, 2B, 3A and 3B are highly divergent among *Picornaviridae* genera [2]. The replication cycle of these viruses rapidly occurs in the cytoplasm with nonstructural viral proteins contained in replication organelles and host cell machinery [2,5].

Seneca Valley Virus Genome

Seneca Valley virus is a small (27-30 nm), non-enveloped virus belonging to the *Senecavirus A* species within the *Picornaviridae* family [6,10]. Three clades have been identified using phylogenetic analysis of SVV VP1: clade I contains the historical strain SVV-001, clade II contains USA SVV strains identified between 1988 and 1997, and clade III contains global SVV strains from Brazil, Canada, China, and the USA identified between 2001 and 2015 (Fig. 2) [11]. In regards to other *Picornaviridae* viruses, *Senecavirus A* is most closely related to the *Cardioviruses* based on complete genome sequence analysis [6,12,13].

In 2008, the SVV-001 genome was determined to contain 7280 nt which is predicted to contain 666 nt of UTR at the 5' end followed by an ORF of 6543 nt encoding a 2181 amino acid polyprotein, a 71 nt 3' UTR and ending with a poly(A) tail [6]. The polyprotein is divided into three polypeptides: P1, P2 and P3 that are further cleaved into eleven proteins in the standard *Picornaviridae* L-4-3-4 layout [7]. The P1 region encodes the structural proteins VP0, VP3 and VP1 that make up the viral capsid while the P2 and P3 regions encode nonstructural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D which are responsible for protein cleavage and viral replication. The VP0 precursor protein is presumed to be cleaved into

VP4 and VP2 due to the start of the P1 region occurring 71 residues upstream of the predicted VP2 sequence [6].

Seneca Valley Virus Proteins Involved in Capsid Formation

The SVV P1 polyprotein is processed by self-cleaving proteases to form individual viral capsid subunit proteins VP1-VP4. The 3C viral protease separates VP0, VP3 and VP1 proteins from each other to form an intermediate protomer. This protomer further assembles into 12 pentamers forming an icosahedral capsid. The VP0 protein is predicted to cleave into VP4 and VP2 during a maturation cleavage involving RNA encapsidation [2]. On the external surface of the viral capsid VP1, VP2 and VP3 proteins are exposed while VP4 is internal [11]. The N-terminal sequences of the three major structural proteins VP1, VP2 and VP3 have been determined with amino acid sequencing. Based on the N-terminal sequences, the predicted cleavage sites of VP4/VP2/VP3/VP1 are Lys/Asp, Gln/Gly and His/Ser, respectively [6].

The SVV 3C protease responsible for cleaving VP0, VP3 and VP1 is a chymotrypsin-like enzyme with a cysteine in place of a serine in the catalytic site [14,15]. Many active-site residues and amino acid motifs of SVV 3C protease are conserved with other known picornavirus 3C three-dimensional structures [11,16]. Predicted cleavage sites for SVV are typical of picornavirus 3C proteases, where cleavage occurs at Gln/Gly, Gln/Ser or Glu/Asn residues [4]. Two atypical cleavage sites include the His/Ser between VP3 and VP1 and Gln/Gln or Gln/Pro between 3B and 3C [6].

Seneca Valley Virus Disease Characteristics

Seneca Valley virus has been associated with swine idiopathic vesicular disease (SIVD) which is characterized by coalescing erosions, ulcerations and vesicular lesions on the snout, oral cavity and coronary bands of the hoof, interdigital area, dewclaws and hoof pads [17-19]. The lesions first appear as swollen areas that evolve to vesicles, which rupture quickly and form ulcers that may be covered by a serofibrinous exudate [11]. Ulcers begin healing within seven days and generally healed in two weeks. Scarring is possible if lesions are severe, but is not observed in all cases. Other clinical signs from SVV outbreaks have included crusting and sloughing of the hoof wall as well as lameness [11,20]. No other gross or microscopic lesions have been observed in affected animals [11]. Other general signs are fever, lethargy and anorexia [11,19]. In recent SVV outbreaks, neonatal piglets 1 to 4 days of age experienced higher rates of mortality, severe diarrhea, dehydration and lethargy [19,21].

While there has been an increase in SVV-associated SIVD, it is not a debilitating disease and mild compared to other foreign vesicular diseases [11,22]. The major risk factor of SVV is the indistinguishable clinical signs it shares with vesicular foreign animal diseases Foot-and-mouth disease (FMD), Swine vesicular disease (SVD), vesicular stomatitis (VS) and vesicular exanthema of swine (VE), not the disease itself [11,19,22]. It also shares clinical signs with porcine enterovirus group III infection, parvovirus infection, mycotoxicosis, chemical burns and photodermatitis associated with the consumption of celery, parsnips, or carrots infested with the fungus *Sclerotinia sclerotiorum* [22].

Seneca Valley virus clinical signs could have a large impact on the pork industry due to the similarity to FMD, which is on the Office International des Epizooties (OIE) disease list. Clinical signs of vesicular lesions must be reported to regulatory authorities so an

investigation can be initiated. Samples are collected for testing to rule out more debilitating diseases such as FMD. During the investigation, animal movement is banned, human movement is restricted and animals may be culled to reduce the risk of spreading disease. Besides the confounding clinical signs of SVV, lameness caused by lesions on the coronary bands can also decrease eating leading to a decrease in average daily gain (ADG), decrease in final market weight, or increase in days to market which all affect profitability.

The majority of SVV cases occur between spring and fall months [22]. In general, transmission of picornaviruses is linked to direct contact with infected individuals, fecal-oral, fomite or airborne routes [2,17]. A specific route of transmission for SVV has not been identified, but general biosecurity measures should be enforced to reduce potential introduction of the virus [17]. Rodents are suspected to be possible carriers due to neutralizing antibodies detected previously in mice [12]. Rodents have also been implicated as reservoirs for encephalomyocarditis virus (EMCV) which belongs to the closely related *Cardiovirus* genus [23]. Seneca Valley virus has been identified in healthy pigs displaying no clinical signs of infection; however, experimental studies with SVV obtained from infected pigs have produced vesicular disease [24-26].

History of Seneca Valley Virus

In 2002, SVV-001 was isolated at Genetic Therapy Inc. near Seneca Creek State Park (Gaithersburg, MD, USA) where it was found accidentally as a cell culture contaminant [6,11,12]. It is believed to have originated from cell-culture medium from either contaminated porcine trypsin or fetal bovine serum. Since the sequencing of this prototype strain, at least twelve virus isolates from pigs in the United States have been submitted to the National

Veterinary Services Laboratory and identified to be serologically similar [6]. Between 1988 and 2005, seven isolates were identified as SVV using pan-picornavirus RT-PCR [12]. Isolates were collected from Minnesota, North Carolina, Iowa, New Jersey, Illinois, Louisiana, South Dakota and California. Seneca Valley virus is a relatively new virus in swine herds in the U.S. based on evolutionary analyses of the virus. It has also been identified in other countries such as Canada, China and Brazil[10,17].

Generally, interest in SVV has been directed more towards its oncolytic properties for human cancer treatments rather than its disease etiology in pigs [27-33]. Oncolytic viruses like SVV are replication-competent viruses that selectively cause cytotoxicity in cancer cells without excessively damaging normal tissues [11,29,32]. A great deal of research has been done with SVV in treating neuroendocrine cancers including clinical trials [34,35]. Although most of the research for SVV is focused on its oncolytic properties, there have been some clinical reports of disease in swine linked to SVV.

In 2007, a group of 187 pigs brought to Minnesota from Canada had twelve pigs with apparent lesions indicative of vesicular disease and at least 80% were considered lame [22]. Tests for FMDV, SVD, VSV and VEV were all negative. The presence of porcine circovirus and porcine enterovirus were reported by the United States Department of Agriculture (USDA) while SVV was discovered soon after with further testing. In addition, a boar in Indiana presented clinical signs for vesicular disease and tested positive for SVV while other vesicular diseases were ruled out in 2010 [18]. Seneca Valley virus was also confirmed in three cases by a swine veterinarian in North Carolina in 2012 by RT-PCR and virus isolation [36]. However, due to these low numbers of identified cases in the past there has been little interest in SVV until recent increased outbreaks and implications with SIVD.

Current Scenario with Seneca Valley Virus

There has been a spike in SVV cases in several different countries around the world including the US in the last two to three years leading swine veterinarians to classify SVV as an emerging infectious disease [37]. In the past three years, SVV has been confirmed in Brazil, China and Thailand by RT-PCR of RNA extracted from field samples [7,10,38]. In the US, three cases in Iowa and one case from South Dakota were submitted for swine vesicular disease and tested positive for SVV [39]. These recent cases prompted Veterinary Diagnostic Labs at Iowa State University and University of Minnesota to retrospectively test approximately 1000 oral fluid samples from swine not exhibiting clinical signs of SVV [37]. Samples from numerous states tested PCR positive indicating widespread areas of infection [37].

The current circulating SVV strains are more closely related to each other than to historical strains, and can be categorized into two groups with one containing relatively mild strains that display vesicular lesions and another with more aggressive strains associated with lameness and piglet mortality [39,40]. In November 2014, farms in Brazil reported pigs with vesicles and lesions on the snouts and coronary bands, neonatal pig mortality and self-limiting outbreaks lasting 1-2 weeks [21]. A unique observation of the SVV cases in Brazil was the neonatal piglet mortality recently described as epidemic transient neonatal losses (ETNL) syndrome. Piglet mortality in the first four days of life was between 30-70% with SVV identified by PCR in the lung, heart, liver, spleen, kidney and intestinal tissues [21]. Samples were submitted to the University of Minnesota Veterinary Diagnostic Laboratory from vesicles and sera of pigs displaying signs of vesicular disease. Three complete genome sequences were constructed from the samples and showed 99.5% nucleotide identity [21].

Based on phylogenetic trees, these sequences were most closely related to a Canadian strain[21].

In July and August of 2015, three Iowa swine farms had several cases of vesicular disease in sows showing lesions on coronary bands of the hoof and nostrils lasting for no more than 10 days [19,20,41]. An increase in mortality to 30-40% was observed in neonate piglets which paralleled the mortality rates reported with the SVV cases in Brazil [19,20]. One field study determined that SVV seropositive sows could transfer maternal antibodies to their offspring. They also noted SVV-VP1 IgG antibodies in sows increased the first three weeks following clinical signs of outbreak in conjunction with decreased detectable levels of SVV in the sera. These finding suggest possible neutralizing capabilities of the SVV-VP1 antibodies due to reduced viremia in the presence of the SVV-VP1 antibodies [19]. In other *Picornaviridae* viruses such as FMDV and Enterovirus 71 (EV71), VP1 is described as the most immunogenic capsid protein due to several neutralizing antibodies identified against epitopes located on it [9,19,42-44].

To date, all SVV-positive vesicular disease outbreaks have tested negative for other vesicular diseases. The increased number of cases for SVV-associated idiopathic vesicular disease in swine triggered Iowa State University Veterinary Diagnostic Lab (ISU VDL) to test other submitted samples describing clinical signs associated with the presence of SVV. From July 2015 to September 2017, more than 230 cases of SVV have been confirmed across all swine production sites, as well as, exhibition pigs and a truck wash [45,46]. The USDA has labeled SVV as a new, emerging swine disease that is active in the industry [40], and the Center for Food Security and Public Health has stated that methods for preventing and controlling SVV are lacking with no vaccines currently available [17].

General Overview of Vaccine Approaches in the Animal Health Industry

The concept for vaccination gained popularity in the late 18th century when Edward Jenner, the pioneer of vaccines, created the world's first vaccine for smallpox [47]. Jenner's discovery was instrumental in defining the fundamental principles for the development of human and animal vaccines. Vaccines are the most effective way to control disease today [48]. Conventional methods consist of inactivated and live attenuated vaccines. Inactivated virus vaccines are prepared by inactivating the virus with heat, chemicals or radiation and may be combined with an adjuvant to boost the immune response [49-51]. This type of vaccine is typically safer and more stable than the live attenuated vaccine [52]. However, inactivated vaccines generally stimulate a weaker immune response compared to live attenuated vaccines and may require booster shots to achieve optimal immunity [53,54].

Live attenuated vaccines are weakened versions of the natural disease-causing virus; therefore, they are also the most similar to the natural virus. Attenuation is commonly achieved by serial cell-culture passage of the virus in cell cultures derived from heterologous species. This encourages adaptations that render the virus unable to induce clinical disease in the natural host [49]. Chemical mutagenesis reagents can also be added to increase mutations during cell-culture passage. The advantage of live attenuated vaccines is the strong cellular and humoral host responses elicited against viral infection [54]. However, there is the risk for the vaccine virus to mutate and revert to virulence [53]. This is a major concern for those diseases that can have huge impacts on the animal industry such as FMD. With the increase in molecular research, opportunities to address the concerns of conventional vaccines show great potential for innovative approaches to vaccine design.

Innovative technology vaccines such as DNA vaccines, subunit vaccines and recombinant vector vaccines are becoming increasingly important with the need to have vaccines that are safe and effective [50]. In addition to the safety and efficacy of vaccines, it must be kept in mind that production costs and ease of administering vaccines are also major factors in the animal industry. Potential for differentiation of infected from vaccinated animals (DIVA) is an approach DNA, subunit and recombinant vector vaccines can utilize. This technique is often not available when using inactivated or live attenuated vaccines [50]. This aids as a tool for animal caretakers and veterinarians for disease surveillance and eradication programs with vaccine use. These alternative technology vaccines also allow for flexibility and variety of vaccine design with DNA manipulation providing opportunities to express single and multiple antigens from the same or different pathogens.

DNA vaccines are simple and inexpensive to produce while providing target specificity of immune responses. DNA vaccines are designed to incorporate specific antigenic genes of a virus that the immune system responds to as if induced by the replication of a live pathogen [55]. Immunization with a DNA vaccine converts the host cells into vaccine antigen-making factories, translating the viral genes then displaying the antigens on their cell surface or secreting them into the extracellular space resulting in a strong antibody and cellular response [55]. Two licensed veterinary DNA vaccines are West Nile-Innovator® DNA for horses and APEX-IHN® approved by the Canadian Food Inspection Agency to prevent Infectious Haematopoietic Necrosis (IHN) in farm-raised salmon [55]. In a mouse model, a DNA vaccine containing the P12A3C genome of EMCV elicited neutralizing antibodies, protected against challenge and was comparable to a commercial vaccine [56]. There was also evidence of increased cytokine levels for IL-2, TNF-α, GM-CSF, IL-4 and IL-10 [56]. Studies

conducted in swine with vaccines encoding a surface glycoprotein for classical swine fever virus (CSFV) and pseudorabies virus (PRV) resulted in protective immune responses as well[55]. The major disadvantage of DNA vaccines is the large amounts of DNA needed for an effective response in animals [55]. Improvements in plasmid uptake, adjuvants, different routes and modes of administration are all approaches that are being investigated to improve the efficacy of DNA vaccines [55].

Subunit vaccines are made up of specific antigens that have been produced and purified from the virus or generated using DNA technology prior to vaccine formulation [49]. Subunit antigens are administered without viral replication occurring in the host [55]. In general, immunogenicity derived from subunit vaccines may require higher doses, booster administrations and adjuvants to achieve protection comparable to that induced by live attenuated vaccines [43,48]. One of the first examples of this technology was in the 1970's when scientists discovered that a vaccine made up of a single key protein of FMDV could potentially protect against the disease [57]. In 1981, Kleid and colleagues were able to express the VP3 of FMDV in *Escherichia coli* and prepare the world's first ever genetically engineered vaccine for use in cattle and swine [42].

Recombinant viral vector vaccines use an attenuated virus to present the antigen of interest to the animal to evoke an immune response [49]. Research has shown the recombinant adenovirus vector containing picornavirus EMCV P1 with 2A and 3C proteases produced neutralizing antibodies against VP1 in mice and protected against challenge [23]. The first licensed vaccines using this technology protect against hepatitis B Virus (HBV) and human papillomavirus (HPV) [58]. Both vaccines are very successful and often used as the gold standard for comparison with other recombinant vector vaccines. This type of genetically

engineered vaccine has combined many advantages of the conventional and innovative technologies making it a highly sought after method for producing safe and efficacious vaccines [50].

VLP Vaccines

Another group of genetically engineered vaccines are virus-like particles (VLPs) that form empty capsids resembling the appearance of native virus externally, but do not contain genetic material [55,59]. Virus-like particles present an array of relevant epitopes similar to those of infectious virions [60,61]. Continuous and discontinuous antigenic sites presented by VLPs elicit immune responses comparable to killed virus vaccines [9,62]. Virus-like particles can induce strong humoral and cellular immune responses due to the repetitive antigenic proteins that lead to crosslinking of B cell immunoglobulin receptors and B cell activation [48]. The antigens are also taken up by dendritic cells which in turn stimulate $CD4^+$ cells to react to the VLP [48,55]. Several studies have demonstrated these strong humoral and cellular immune responses in different animal species with administration of VLPs through various routes such as intranasal, intramuscular and intraperitoneal [61,63]. Antigens from picornaviruses such as FMDV, EMCV, EV71, coxsackievirus A (CVA), and hepatitis A (HAV) have been expressed as VLPs and shown to confer neutralization and/or protection[23,44,62,64-66].

Several features of VLP vaccine technology deem it safer compared to traditional inactivated and live attenuated vaccines [61,67]. These include a reduced risk of adverse reactions, lack of replication, no reversion to virulence and no possibility of vaccine-associated outbreaks. Virus-like particle vaccines also have DIVA marker potential to

differentiate infected animals from vaccinated animals since antibodies are not generated against internal and non-structural viral proteins [55]. Compared to DNA and subunit vaccines, the strong immune responses elicited by VLPs make it a preferred vaccine platform [49,67]. The capability to quickly switch out strain sequences in response to evolving viruses is also an advantageous feature of the VLP vaccine platform. Additionally, the VLP molecular platform can be modified to display viral epitopes of a heterologous virus boosting immunogenicity of the specific epitope and creating a multivalent vaccine [44,55,58,61,67].

Expression systems that generate VLPs have different characteristics to take into consideration when choosing the production platform. The *E.coli* expression system is the most widely used platform for recombinant proteins due to easy scale-up and high expression yields [63,68,69]. However, bacteria lack the ability for proteins to undergo post-translational modifications (PTM) making it the least versatile of the VLP expression systems [48]. For this reason, it works best with simple, non-envelope proteins [44]. The other expression systems are eukaryotic which allow for PTM at varying levels. Yeast provides easy scale-up and high expression yields of VLPs, and in addition allows PTM to occur [70-72]. However, the sugar residues used in glycosylation by yeasts are different from those used by mammalian cells. The mammalian cell expression system's greatest advantages are the ability for recombinant proteins to undergo PTM and the assembly of complex, multi-protein VLPs [73,74]. Conversely, the production cost of using mammalian cells is the highest of the expression platforms. Plant biotechnology has opened up the possibility of expressing VLPs in plants with low cost processing, easy scale-up and increased safety since plants are free of animal diseases [55,75,76]. A plant-based system is similar to yeast, mammalian, and insect expression systems with the ability to express VLPs from non-envelope and envelope proteins that

undergo PTM [48]. Plant-based systems also provide a means of administering the vaccine by direct ingestion of the plant. Despite the easy scale-up and ease of administration for plant-based VLPs, issues with VLP assembly, stability, antigen degradation during *in vivo* delivery and low expression levels need to be addressed [55,57,76]. The baculovirus expression vector system (BEVS) has been described as the most powerful and versatile eukaryotic expression system for producing complex, multi-protein VLPs [77]. The BEVS uses insect cells infected by recombinant baculovirus to express antigens of interest [67]. Insect cells have an accelerated metabolism ideal for producing high protein yields [55]. The BEVS also provides production versatility, scalability, efficiency and speed of vaccine development [55,67].

Baculovirus Biology

Baculoviruses belong to the *Baculoviridae* family, and are large (80-200Kb), double-stranded, circular DNA viruses that infect many species of insects [77,78]. The enveloped, rod-shaped nucleocapsid can accommodate large amounts of foreign DNA which led to the wide use of these viruses as recombinant vectors [67,77-79]. Originally, the wild-type baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) was used for co-transfection with recombinant transfer vectors [79]. This method took a considerable amount of time and effort for screening and selecting recombinant baculovirus particles with only 0.1% recombination frequency [77,80]. This led to the modification of AcNPV and *Bombyx mori* nucleopolyhedrovirus genomes for research purposes to generate insect cell-derived vaccines with AcNPV being the most widely used [55,59,78].

Infectious AcNPV enters susceptible insect cells by facilitated endocytosis or fusion. Viral DNA is uncoated in the nucleus and replication starts to take place six hours after the

baculovirus infects the host cell [77]. Occlusion-derived viruses (ODV) are important for horizontal transmission of disease and are assembled in a crystalline protein matrix within the nucleus [78]. In wild-type AcNPV the ODV develop during the very late phase of gene expression [78]. This is usually around three days post infection and continue to accumulate until five or six days post infection [77]. These occluded virus particles are primarily made up of the polyhedrin protein, and are released by cell lysis [77,78]. Increasing amounts of polyhedrin protein during the very late phase of infection may account for 30-50% of the total insect cell protein [77]. Secondary infection is denoted by extracellular virus particles that bud from the cell membrane of infected cells and are referred to as budded viruses (BV) [78]. They are produced during the late phase of gene expression and are responsible for cell-to-cell infection [78]. The BV is exploited in the BEVS for foreign protein expression.

Harnessing the Baculovirus Platform Technology for protein expression

The BEVS has several features that make it the workhorse of expression systems. It is simple to use, allows for large foreign protein inserts, multiple gene expression, signal peptide cleavage, intron splicing, nuclear transport, functional proteins, and PTM [59,77,78,81]. The baculovirus genome is modified with restriction sites that are utilized to delete sections of essential genes. This linearizes the baculovirus genome facilitating the homologous recombination with transfer vectors containing a gene or genes of interest. This in turn restores the lethally deleted virus and creates a viable recombinant baculovirus [77,80,82]. Transfer vectors are most often utilized for insertion of heterologous genes as the large size of the baculovirus genome makes *in vitro* manipulation difficult [77]. Homologous recombination of the baculovirus and plasmid occurs in insect cells that are susceptible to

baculovirus infection such as Sf9 and Sf21 cells. These cells were originally established from ovarian tissues of *Spodoptera frugiperda* larvae [77,78]. After 3-5 days the homologous recombined baculovirus is harvested with 99% efficiency [77].

The polyhedrin gene is the primary location for heterologous gene insertion because the protein is produced in large amounts in the native virus. It is also nonessential for the baculovirus life cycle in tissue culture [77-79]. Many baculovirus transfer vectors have been designed with the AcNPV genome to switch out the polyhedrin gene for a heterologous gene. The pVL1392 and pVL1393 vectors have been used extensively with the baculovirus platform and are based on the polyhedrin locus [77]. These vectors contain an *E.coli* origin of replication, an antibiotic resistance marker, the polyhedrin gene promoter region, a multiple cloning site to insert the gene or genes of interest, and AcNPV sequence flanking the cloning site to enable homologous recombination [77].

Although the polyhedrin locus is most extensively used, other loci are capable of modification for heterologous protein insertion. The decision to use different loci is determined based on whether the timing of protein expression during the viral infection cycle is important. Baculovirus-encoded promoters can be divided into four different types based on the chronological order in which they are activated [77,78]. Immediate early promoters are activated by and control early viral transcription factors while delayed early promoters control genes necessary for viral replication, and thus are activated before viral DNA synthesis occurs [77,78]. The immediate early or early promoters are typically not used for heterologous genes due to their essential roles in the virus life cycle and their overall weak expression during the baculovirus infection cycle [83]. Late promoters are active during and after viral DNA synthesis and control genes necessary to assemble virus particles [83]. Very

late promoters are not activated until after viral assembly is complete and control genes responsible for the formation of occlusion bodies and cell lysis [77,78]. The late and very late promoters are commonly used in baculovirus transfer vector design because they drive strong protein expression [67,77-79]. The polyhedrin and p10 promotors are examples of very late promoters that can be utilized together in transfer vectors to express two different proteins simultaneously [81,84].

Recombinant protein is produced by the baculovirus taking over insect cell machinery and shutting off host gene expression [77,78]. The recombinant virus continues to infect additional insect cells, which results in additional recombinant protein produced. Baculovirus-expressed proteins are typically recovered from the same sub-cellular compartments as the native protein unless additional sequence is added such as a signal sequence that direct the protein to be secreted [77]. Recombinant protein production can reach up to 30%-50% of the total insect cell protein produced, but amounts vary based on the properties of each individual protein and number of promoters utilized [77,85].

One disadvantage of the BEVS is the inherent coproduction of baculovirus particles, which can affect antigen yields. Baculovirus particles also may interfere with the VLP-specific immune response of the host possibly decreasing vaccine effectiveness [59]. Either a chemical inactivation has to be performed in order to eliminate baculovirus infectivity, or several downstream processing steps must be performed [67]. Both methods have the potential to impair VLP quality, quantity and stability [57].

Successful application of the BEVS among the *Picornaviridae*

The BEVS system is an ideal expression system for vaccine development for picornaviruses given they naturally form immunogenic, empty viral capsids [86]. Several picornaviruses including EV71, *Poliovirus* and FMDV exhibit empty viral particle capsids during natural infection [9,87-89]. These empty capsids do not contain viral genomic RNA and therefore are non-infectious. In several studies, these naturally occurring empty capsids have been shown to be antigenically similar in immunological and serological tests when compared to native virus containing RNA [65,86,87,90]. Expressing VLPs using the BEVS for vaccine development provides options to control and manipulate the design of the empty capsid for increased stability, immunogenicity, expression levels, and opportunities for chimeric VLPs to protect against different strains or types of viruses [59]. Several approaches for expressing VLPs in the baculovirus system have been evaluated for different picornaviruses. However, to the best of my knowledge no published work to date has been shown successfully expressing SVV VLPs.

One approach to baculovirus construct design is to insert the complete ORF of a virus for VLP expression. The translation of HAV and poliovirus ORFs has led to protein VLP assembly that has elicited neutralizing antibodies in mice [66,91]. Several other baculovirus constructs have been designed to co-express only the picornavirus genes that encode the capsid proteins and picornavirus proteases such as 2A, 3C and 3CD proteases. The inclusion of the 3D gene with the 3C depends on the specific virus as some viruses like poliovirus and coxsackievirus need the 3D to efficiently cleave the P1 polyprotein, while in contrast FMDV can efficiently cleave the P1 independently [92]. Baculovirus constructs with this approach have produced VLPs that stimulate immune responses and the production of neutralizing

antibodies in mice, guinea pigs, swine, cattle, and macaque monkeys [8,61,62,64,65,93-97]. Humoral immune responses induced by EV71 VLPs in mice and macaque monkeys included high neutralizing antibody titers, while Th_1 and Th_2 immune responses have produced high levels of IFN-γ, IL-2 and IL-4 [64,94]. Neutralizing antibodies have also been shown to protect against lethal challenges by passive transfer of antisera and active immunization in mice for EV71, CVA6 and CVA16 infections through baculovirus-expressed VLPs [64,93,95].

Virus-like particles have also been produced by simultaneous transfection of insect cells with two baculovirus constructs encoding recombinant P1 and 3CD proteins separately for EV71 [9,98,99] . In 2003, EV71 VLPs resembling native EV71 aggregates were constructed for the first time using this method [98]. A study was then conducted for EV71 VLPs comparing two separate recombinant baculoviruses expressing P1 and 3CD independently to that of a single recombinant baculovirus co-expressing P1 and 3CD [9]. The EV71 VLPs with the single recombinant baculovirus co-expressing P1 and 3CD produced more VLPs than that expressed via simultaneous infection with two separate baculoviruses expressing the P1 or 3CD respectively [9]. Nonetheless, the VLPs from simultaneous infection with two separate baculoviruses produced neutralizing antibodies against different strains of EV71 in immunized mice similar to the co-expressed P13CD baculovirus constructs [99]. Another construct design used the BEVS and codon-optimized FMDV genes for insect cells. The 3C protease was omitted and instead the 2A protease was utilized to cleave capsid proteins VP1 and VP3 to produce VLPs along with VP0 expressed under a second promoter [100].

These studies prove that VLP assembly of picornaviruses can occur in the BEVS. However, no research has been published to date on attempts of SVV recombinant capsid protein expression with VLP assembly. The BEVS is ideal for assembling SVV VLPs

because of its efficiency of expressing complex, multi-protein VLPs. The BEVS also allows for rapid modifications of the genome to evaluate different variations. Exploiting the knowledge gained from these previous works, the BEVS was utilized to engineer baculovirus constructs encoding SVV viral proteins. These constructs were evaluated for expression of SVV recombinant capsid proteins and VLP assembly. The findings of this research may further support picornavirus VLP assembly strategies and understanding of SVV viral capsid formation.

CHAPTER 3: EXPRESSION AND PURIFICATION OF SENECA VALLEY VIRUS RECOMBINANT CAPSID PROTEINS FROM BACULOVIRUS-INFECTED INSECT CELLS

Introduction

The increase of clinical manifestations of SVV in the last three years and the resemblance to FMD has increased biosecurity concerns in regions where FMDV is not present. Negligence in reporting clinical signs of SVV could provide foreign vesicular diseases an opportunity to spread rapidly through swine herds before being detected. A vaccine against SVV is one possibility to reduce this dangerous threat to the US pork industry.

Baculovirus-expressed picornavirus VLPs have demonstrated several key traits that are required for efficacious vaccines. Baculovirus-expressed picornavirus VLPs have elicited virus-specific neutralizing antibodies, cellular and humoral immune responses, passive immunity and protection against virus challenge [62, 63, 71, 75-79]. A VLP based vaccine utilizing the BEVS could provide an effective and safe vaccine with DIVA potential for SVV. By decreasing the prevalence of vesicle presentation caused by SVV, the vaccine could support rapid identification of devastating foreign diseases of swine exhibiting similar clinical signs.

Seneca Valley virus encodes a single polyprotein that contains structural proteins VP1, VP2, VP3 and VP4 that create the viral capsid when processed into individual subunits by viral proteases, as in many other picornaviruses [11]. An empty viral capsid structure has been identified for several picornaviruses during the native infection process and has been

shown to be stable under certain conditions [86,101]. Virus-like particles that are near identical to the native empty viral capsids have been assembled with the BEVS. Based on previous published literature of picornavirus VLP assembly, an initial design for SVV baculovirus constructs was modeled after FMDV constructs [84,97]. The transfer vector contained the SVV P1 region, portions of 2B and 3B, and the self-cleaving proteases 2A and 3C. The herein described SVV baculovirus constructs were evaluated for VLP formation to determine their suitability to serve as potential recombinant subviral vaccine candidates.

Materials and Methods

Design and construction of SVVP13C recombinant capsid protein plasmids

Two gene sequences were designed and ordered in the pUCIDT-Amp vector from Integrated DNA Technologies, Inc. (IDT) based on the resultant consensus sequence of SVV 26, SVV 27, SVV 29, SVV 30 and SVV 49 received from Kansas State University. The SVVP13C gene ordered was native consensus sequence of the full length P1 polyprotein with 2A and partial 2B and 3B sequences connecting the P1 polyprotein and 3C self-cleaving protease. The SVVP13C-CO gene ordered had the P1, 2A and 2B sequences codon-optimized for Sf9 insect cells using the IDT Codon Optimization Tool while the 3B and 3C remained as native sequences. Both genes contain a Kozak sequence before the start codon, as well as BamHI and NotI restriction sites at the 5' and 3' ends, respectively. The SVVP13C and SVVP13C-CO gene inserts were excised from pUCIDT-AMP-SVVP13C and pUCIDT-AMP-SVVP13C-CO plasmids by BamHI and NotI digestion, respectively, and ligated into the pVL1393 vector (Fig. 3).

Design and construction of WSSV sIRES-split his-tagged SVVP1 with SVV3C recombinant capsid protein plasmids

PCR amplification was performed with the SVVP13C and SVVP13C-CO genes in the pUCIDT-Amp vectors to amplify the SVVP1 or SVVP1CO sequence with a C-terminal 6x-His tag and the SVV3C sequence (See Table 1 for primers). The SVVP1, SVVP1-CO and SVV3C PCR products were independently ligated into pCR-BluntII-TOPO. The SVV3C insert was excised from pCR-BluntII-TOPO-SVV3C by SpeI and SacI digestion and ligated into the pORB vector following the WSSV sIRES site to generate pORB-MCS1-sIRES-SVV3C. Next, SVVP1-His or SVVP1CO-His sequences purified from pCR-BluntII-TOPO were ligated into the pORB-MCS1-sIRES-SVV3C plasmid. The pORB-SVVP1-His-sIRES-SVV3C and pORB-SVVP1CO-His-sIRES-SVV3C were submitted to ISU DNA Facility for sequencing (Fig. 4). The pORB-SVVP1-His-sIRES-SVV3C had two nucleotide substitutions in its insert sequence. One nucleotide substitution was in the VP1 at position 2404 from G to T that caused a polar to polar amino acid change of glycine to cysteine. The second substitution was in the SVV 3C sequence of both plasmids submitted for sequencing at nucleotide position 133 from C to A causing a non-polar to non-polar amino acid change of leucine to isoleucine. These changes are not expected to alter the predicted structure based on analysis with the MOE program (Molecular Operating Environment [MOE], 2013.08; Chemical Computing Group ULC, 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2017).

Design and construction of SVVP13C VP3/VP1 and SVVP13CD recombinant capsid protein plasmids

Primers were designed to mutate the VP3/VP1 cleavage site of the SVV P1 sequence in pUCIDT-AMP-SVVP13C from FH/ST to PQ/GV (See Table 2 for primers) using the

Lightning Quik Site Directed Mutagenesis kit (Stratagene). The pUCIDT-AMP-SVVP13C VP3/VP1 contained the correct mutation sequence and the SVVP13C VP3/VP1 insert was excised and ligated into the pVL1393 vector to produce pVL1393-SVVP13C VP3/VP1 (Fig. 5). Sequence results matched the expected DNA map except for a single point mutation in VP2 at position 946, changing G to T. The nucleotide substitution resulted in a non-polar to non-polar, aromatic amino acid change from valine to phenylalanine. Evaluation of the valine to phenylalanine amino acid change with MOE suggested there would be minimal effect on the expected capsid structure. The second construct designed ligated the SVV3D sequence to the C-terminal end of the SVV3C sequence by overlap extension PCR (OE-PCR) of a SVV3D PCR product from cDNA to a SVVP13C PCR product amplified from pUCIDT-AMP-SVVP13C to generate pCR-BluntII-TOPO-SVVP13CD (See Table 2 for primer sequence). Due to an internal BamHI restriction site in the SVV3D sequence, BamHI digestion of the SVVP13CD insert resulted in two pieces, which were subsequently ligated into the pVL1393 vector creating pVL1393-SVVP13CD (Fig. 5).

Expression of the SVV recombinant capsid protein constructs in insect cells

The recombinant SVV capsid constructs in the pVL1393-based plasmids were co-transfected with FlashBAC ULTRA (FBU) baculovirus DNA into Sf9 cells, whereas the pORB-based plasmids were co-transfected with BaculoGold baculovirus DNA into Sf9 insect cells. Both sets of co-transfections used ESCORT transfection reagent per manufacturer's instructions. Cell culture supernatants from the transfected Sf9 cells were harvested and clarified by centrifugation at 1,000xg for 5 min to pellet the cellular debris. The clarified supernatant was collected, 0.2 µM-filtered and stored as the P1 transfection harvest. Sf9 insect cells were used to generate P2 stocks, and the P2 stocks were then used to generate P3

and P4 amplifications of the SVV constructs for protein expression evaluations in SF+ insect cells. The SF+ insect cell line is a derivative of *Spodoptera frugiperda* insect cells. Baculovirus-infected SF+ cells were harvested and clarified at 10,000xg for 10 min at 4°C. The cultures of baculovirus-infected SF+ cells were sampled daily to monitor total cells/mL, viable cells/mL, percent viability and cell diameter by Vi-Cell analysis. Amplifications were harvested when viability was ≤ 30% viability or when viable cells were ≤ $1x10^6$ cells/mL. Additional one mL daily samples were collected to evaluate the progression of protein expression during infection and were processed as described above. Collected supernatant and cell pellet samples were stored at -70°C until evaluation.

Lysis of insect cell pellets to separate soluble and insoluble fractions

SF+ insect cell culture samples were centrifuged to pellet the cells after which the media was removed and the cell pellets were frozen until lysis. Pellets were re-suspended in lysis buffer containing the following: 20mM Tris, 1% Triton X-100, Protease Inhibitor Cocktail for His-tagged Proteins (10µL/mL) and Benzonase (250 units/mL) in de-ionized water with a pH of 7.4. The re-suspended insect cell lysates were vortexed for 10 sec, incubated at room temperature for 5 min, vortexed again for 10 sec than centrifuged at 19,090xg for 10 min at 4°C to pellet insoluble material. The soluble lysates were separated from the insoluble fractions and stored in tubes at -70°C.

Purification of SVV recombinant capsid proteins

Supernatant harvests containing the expressed recombinant SVV capsid proteins were 0.2µM-filtered, dispensed into ultracentrifuge tubes, and centrifuged at 100,000xg for two hours at 4°C to pellet protein and possible VLPs. The clarified supernatant was carefully decanted and the pelleted material was re-suspended in TBS and stored at 4°C.

Discontinuous 10% - 60% sucrose gradients were used to further purify recombinant SVV capsid proteins for the respective constructs. The respective re-suspended materials were added to the top of the gradient and centrifuged at 100,000xg for two hours at 4°C. Fractions from the sucrose gradients were collected equally into tubes (with fraction 1 starting at the top of the gradient surface) and stored at 4°C.

SDS-PAGE & Western blot

SDS-PAGE was performed using the NuPAGE electrophoresis system and 4-12% Bis-Tris MES mini gels. Samples were separated under reducing conditions using 0.05M DTT at 175V for the appropriate time. Gels were stained for total protein using an eStain 2.0 Protein Staining Device or transferred to nitrocellulose membranes using the iBlot system for Western blots. Western blots were performed with α-SVV peptide rabbit polyclonal antibodies (α-SVV VP1-2, α-SVV VP2-2, and α-SVV VP3-1, varying dilutions) and goat anti-rabbit peroxidase-labeled secondary antibody (1:500) by the Snap ID method, utilizing negative control baculovirus antigen in the antibody diluent, and developed using TMB membrane peroxidase substrate.

Dialysis and concentration of purified SVV recombinant capsid proteins

Sucrose gradient fractions containing the recombinant protein as determined by Western blot were pooled together and dispensed into 10,000 molecular weight cut-off (MWCO) or 50,000 MWCO cellulose membrane dialysis cassettes. The dialysis cassette was placed in 3.5L of TBS with a magnetic stir bar, covered, and placed on a stir plate at 4°C for a minimum of 6hrs. The dialysis cassette was then placed into a fresh beaker of 3.5L of TBS, and further dialyzed by stirring overnight or longer. The sample was removed from the dialysis cassette and concentrated, if needed, depending on the volume of the dialyzed

sample. Concentration was performed using a size-exclusion filter unit and centrifugation according to manufacturer's directions until desired sample volume was achieved.

Electron microscope imaging

The sucrose gradient-purified and dialyzed recombinant SVV capsid proteins were evaluated by transmission electron microscopy (TEM) at the USDA National Animal Disease Center (NADC) with Judi Stasko for the visualization of VLPs. The expected size of the icosahedral viral capsid for native SVV is approximately 27nm in diameter.

Results

Expression of SVV capsid proteins in baculovirus-infected insect cells

The BaculoFBU/SVVP13C and BaculoFBU/SVVP13C-CO constructs were used to infect SF+ cells, and samples were collected to evaluate the expression of SVV capsid proteins. Protein bands of expected sizes for VP1, VP2 and VP3 capsid subunits were detected by Western blot with α-SVV P1 subunit-specific antibodies in the supernatant (Fig. 6). The recombinant proteins expressed from the baculovirus-infected insect cells were also similar in size to native SVV capsid proteins. Interestingly, an additional protein band of ~55kDa was detected in the α-SVV VP1 and α-SVV VP3 Western blots. The ~55kDa band was not detected in the native SVV antigen sample or the negative control. The presence of the additional protein of ~55kDa suggests that it may comprise an uncleaved VP3-VP1 protein product and merits further investigation. Figure 6 also provides a comparison of SVV capsid protein expression levels between the native (A) and the codon-optimized (B) SVV DNA sequences in baculovirus-infected SF+ cells. Based on these Western blots, there was no readily apparent difference in SVV capsid protein expression levels between the codon-

optimized BaculoFBU/SVVP13C-CO and the native BaculoFBU/SVVP13C.

The SVV baculovirus constructs that were WSSV sIRES-split his-tagged SVVP1 with the 3C protease placed behind the sIRES did not produce detectable SVV capsid subunit proteins. Consequently, this set of constructs was not further evaulated.

BaculoFBU/SVVP13C-derived SVV capsid subunits do not form VLPs

As a control to be used in determining the expected mobility of BaculoFBU/ SVVP13C-derived SVV capsid proteins in a sucrose gradient, pelleted native SVV virus was separated on a sucrose gradient and analyzed by Western blot (Fig. 7A). The majority of native SVV subunit proteins were detected in fractions five and six after sucrose gradient purification for native SVV. It was anticipated that if VLPs were formed in the baculovirus-infected SF+ cells they would present in a similar or slightly higher range of collected gradient fractions when compared to that of native SVV.

Harvest supernatant from the BaculoFBU/SVVP13C construct was also processed and subjected to sucrose gradient purification. Western blots of the sucrose gradient fractions only detected a small portion of the recombinant SVV VP2 capsid protein in sucrose fraction one (Fig. 7B). VP1 and VP3 were not detected in the sucrose fractions, although a faint protein band at ~55kDa, thought to be uncleaved VP3-VP1, was detected throughout the sucrose fractions by α-SVV VP1 in the Western blot. Recombinant SVV capsid proteins were not detected in fractions five and six in contrast to the detection of expected proteins from native SVV. These results suggest that the capsid subunit proteins expressed in BaculoFBU/SVVP13C-infected insect cells do not form VLPs.

Modification of VP3/VP1 cleavage site

Previous research identifying the amino acids at the cleavage sites of cardioviruses, with which SVV shares the most similarity, indicated the predicted SVV VP3/VP1 cleavage site His/Ser was unusual compared to the cleavage site Gln/Gly shared amongst cardioviruses such as EMCV and *Theilovirus* [6]. The SVV VP3/VP1 cleavage site is also atypical of the primary picornavirus 3C protease cleavage sites Gln/Gly, Gln/Ser and Glu/Asn [4,6,102-104]. Therefore, a mutation was made at the VP3/VP1 interface in the BaculoFBU/SVVP13C construct to exchange the His/Ser amino acid sequence for Gln/Gly generating the BaculoFBU/SVVP13C VP3/VP1 construct.

This new construct was transfected into Sf9 cells and the resulting baculovirus was evaluated in protein expression assessments in a similar fashion as conducted with the BaculoFBU/SVVP13C construct (Fig. 8). A protein band at the expected full length of the SVV P1 polyprotein, ~95kDa, was detected in the BaculoFBU/SVVP13C VP3/VP1-infected insect cell harvest supernatants. These supernatant samples also contained the recombinant capsid proteins VP1, VP2 and VP3 recognized by their respective antibody. Detection of the putative uncleaved VP3-VP1 protein at ~55kDa and cleaved subunits of VP3 and VP1 were of similar proportion as was observed in BaculoFBU/ SVVP13C supernatant Western blots.

Additionally, Western blot evaluations of the sucrose gradient fractions from the harvest supernatant (Fig. 9) were comparable to the results of BaculoFBU/SVVP13C. The α-SVV VP2 Western blot detected the recombinant SVV VP2 capsid protein throughout the sucrose fractions with the majority in fractions one and nine. Subunit VP1 and VP3 proteins were not detected in the sucrose fractions. Although a protein band at ~55kDa that is possibly the VP3-VP1 uncleaved proteins was detected throughout the sucrose fractions of

the α-SVV VP1 Western blot and in sucrose fractions one and nine of the α-SVV VP3 Western blot. All three Western blots had protein bands detected in the very last sucrose fraction that appeared similar to the starting sample of the sucrose gradient. We conclude from these Western blot evaluations that the mutation of the cleavage sequence from His/Ser to Gln/Gly had no effect on the presence of the ~55kDa band hypothesized to be uncleaved VP3-VP1 proteins. Compared to the original construct, there were no increases in the amounts of subunit VP3 and VP1 proteins detected and no VLP formation was observed.

Modification of 3C protease

Enteroviruses which also belong to the *Picornaviridae* family have been shown to utilize the 3CD protease, a precursor to 3C, for efficient cleavage of capsid proteins in the BEVS and other expression systems [9,70,105,106]. The BaculoFBU/SVVP13CD was designed to include the 3D gene after 3C to assess cleavage of recombinant capsid proteins compared to 3C protease alone in the original BaculoFBU/SVVP13C baculovirus construct.

BaculoFBU/SVVP13CD was expressed in SF+ cells, and the supernatant harvest was evaluated by Western blot (Fig. 10). Results similar to the previous protein assessments were observed by the detection of individual VP1, VP2 and VP3 proteins. The suspected uncleaved VP3-VP1 protein at ~55kDa was also present in the α-SVV VP1 and α-SVV VP3 Western blots.

The Western blots of the BaculoFBU/SVVP13CD sucrose gradient fractions showed the majority of the SVV capsid proteins aggregated and pelleted at the bottom of the gradient (Fig. 11). These results were similar to the BaculoFBU/SVVP13C VP3/VP1 sucrose fraction evaluations. In contrast, these results exhibited monomeric SVV VP1, VP2 and VP3 capsid subunit proteins present in sucrose fractions 1 and 2. In addition, the VP1 and VP2 proteins

were detected throughout the sucrose fractions. Detection of SVV VP1 and VP2 proteins throughout the sucrose fractions suggested the possibility of VLP formation.

EM imaging of purified samples containing recombinant capsid proteins

Despite unclear results from the sucrose gradient evaluation of each construct, fractions expected to contain VLPs were evaluated by TEM at the USDA NADC. Pooled sucrose fractions from supernatant harvests BaculoFBU/SVVP13C and BaculoFBU/ SVVP13CD were dialyzed in TBS and concentrated in preparation for TEM imaging. The sample preparations contained high levels of background particulate making it difficult to visualize VLPs clearly by TEM negative staining. Some spherical shapes were sparsely evident in the BaculoFBU/SVVP13CD pooled sucrose fractions that were similar in size to that expected of SVV VLPs. However, insufficient numbers of putative VLP structures made it difficult to confirm their existence. Representative TEM images are shown in the appendix.

Investigating inside the SF+ insect cells for VLP assembly

One hypothesis as to why the individual recombinant SVV viral capsid proteins were detected in the supernatant, but did not form VLPs is that the VLPs might have disassociated shortly after release into the supernatant. The dissociation could be due to the low pH of the insect cell media or degradation by proteases released during the baculovirus infection. To test this hypothesis, cell pellet samples from Day 3 of BaculoFBU/SVVP13C, BaculoFBU/SVVP13C VP3/VP1 and BaculoFBU/ SVVP13CD infections were lysed in physiological pH buffer. The soluble protein fractions were obtained from the lysed material for evaluation of recombinant SVV subunit proteins by Western blot (Fig. 12). Day 3 was chosen because viral infection was actively ongoing, as indicated by swelling of the cells, but

the viability of the cells was still relatively high as many of the baculovirus-infected cells had not yet lysed.

As expected, there were detectable levels of subunit capsid proteins VP1, VP2 and VP3 in the Day 3 soluble fractions. An ~55kDa band suspected to be uncleaved VP3-VP1 proteins was also detected in the α-SVV VP1 and α-SVV VP3 Western blots as observed in previous Western blot evaluations. Day 3 soluble fraction samples were sucrose gradient purified and evaluated by Western blot to observe if VLPs were present in the cells before lysis (Fig. 13).

The sucrose fractions from the Day 3 soluble fractions had comparable results with the supernatant harvest sucrose fractions from insect cells infected with BaculoFBU/ SVVP13C, BaculoFBU/SVVP13C VP3/VP1 and BaculoFBU/SVVP13CD, respectively. The SVV recombinant capsid proteins were detected mostly in fractions one and two and/or pelleted to the bottom suggesting the SVV subunit proteins inside the insect cells do not form VLPs prior to cell lysis. Transmission electron microscopy was performed on the pooled sucrose fractions 2-4 of BaculoFBU/SVVP13CD Day 3 soluble sample and the pooled sucrose fractions 2-3 of BaculoFBU/SVVP13C VP3/VP1 Day 3 soluble sample (See Appendix for images). Results were inconclusive for VLP assembly for both samples due to high levels of background particulate present.

Discussion

There has been an increase in recent years in SVV cases reporting SIVD clinical signs that are indistinguishable from foreign animal diseases such as FMD and SVD. In response to the increased detection of clinical manifestations of SVV, a VLP-based vaccine would be

very useful. It could deter complacency in reporting SVV vesicles and foreign swine vesicular diseases entering the US undetected. There is no published work to date on VLP assembly for SVV; therefore, the initial SVV baculovirus construct was designed based on successful VLP constructs produced for other picornaviruses [9,62,64,91,98,107]. However, due to ineffective VLP formation in the initial construct, modifications were introduced to explore alternative constructs for VLP assembly. Baculovirus constructs encoded the full-length SVV P1 polyprotein, partial sequences of 2B and 3B, and the 2A and 3C proteases. The SVV 3C protease was incorporated for self-cleavage of the expressed P1 polyprotein into individual protein subunits. Recombinant SVV capsid proteins VP1, VP2 and VP3 were expressed to some extent as fully cleaved proteins and detected by α-SVV peptide antibodies in Western blot. However, VLPs were not detected based on sucrose gradient purification and EM imaging.

An interesting discovery in BaculoFBU/SVVP13C Western blots detected with α-SVV VP1 and α-SVV VP3 antibodies was another protein band identified at approximately 55kDa that was generated in the baculovirus-infected SF+ cells, but not in the native virus. The 55kDa band corresponds to the expected size of uncleaved VP3-VP1 proteins. It was not detected in the α-SVV VP2 Western blot further leading us to suspect it may be a protein specific to VP3 and VP1. This protein band may be an indication as to why VLP formation is hindered in the baculovirus-infected insect cells.

The additional baculovirus constructs were designed to evaluate the cleavage site of VP3 and VP1 in attempts to increase levels of cleaved VP1 and VP3 subunit proteins. Western blot results determined there was no change in the amount of the individual expressed VP3 and VP1 proteins among any of the baculovirus constructs. In addition, the

approximate 55kDa protein band suspected as the VP3-VP1 fusion protein was still detected in the Western blots with α-SVV VP1 and α-SVV VP3 antibodies, and it did not appear to change in the amount expressed.

Specifically, the 55kDA protein band suspected to be VP3-VP1 uncleaved proteins was evaluated for its decreased detection with corresponding expectations of increased detection of cleaved VP3 and VP1 capsid proteins. Conversely, the 55kDa band was still detected in Western blots at comparable levels as the original SVV baculovirus construct. N-terminal sequencing or mass spectrometry could be performed to confirm if the 55kDa band is indeed uncleaved VP3-VP1 proteins.

In Western blot evaluations of sucrose gradient fractions, the recombinant SVV capsid proteins were detected in the first few fractions and/or in the last fraction of the gradient. The presence of proteins in these fractions led us to determine that they remained as non-associated monomers or formed large aggregates indicative of misfolded or misassembled proteins, respectively. These findings could also be the result of an insufficient amount of subunit proteins being expressed resulting in unmet threshold limits for capsid protein concentrations required for VLP formation [108-110].

An aspect that was not evaluated in this research is the impact of picornaviral 3C proteases on host cellular proteins. Some 3C proteases have been shown to cleave important host cell proteins leading to apoptosis [102]. For example, 3C proteases cleave translation initiation factors that control cell survival. These effects of innate 3C activity could affect baculovirus-infected insect cells as well and hinder SVV gene expression in the BEVS [102,111]. In the full SVV genome, the SVV 3C protease targets twice as many predicted cleavage sites than the number of cleavage sites present in the baculovirus constructs [6]. Therefore, normal

expression of the 3C protease required in a native infection to cleave all these sites may not be necessary in baculovirus transfection. The potential excess 3C protease could be prompting proteolytic activity of SF+ proteins in turn affecting capsid protein expression and VLP formation. If 3C-based proteolytic activity within the SF+ cells is reducing capsid protein expression, the present expression levels may not be enough to meet the threshold for efficient SVV VLP assembly. Research into SVV 3C and 3CD protease expression levels using the BEVS should be conducted to get a better understanding of their effect on protein expression in baculovirus-infected insect cells.

Previous research with similarly designed FMDV constructs revealed cell toxicity attributed to 3C expression. Results showed a decrease in FMDV 3C protease expression led to increased amounts of cleaved capsid proteins and VLP formation [107,111]. In a separate experiment, a dual promoter baculovirus vector expressing the EV71 P1 region under the polyhedrin promoter and the 3C protease under a less active promoter also improved VLP yields [112]. This study suggests reducing the level of 3C expression in a dual promoter-based baculovirus vector could have improved EV71 capsid protein production due to reduced competition between P1 and 3C expression when under the same promoter. This prospect is in addition to the 3C protease causing cell toxicity as stated in the FMDV study. In comparison to the EV71 dual promoter baculovirus vector results, an alternate study with FMDV investigated strategies for FMDV 3C expressed under the same or separate promoter as the P1-2A sequence in the BEVS. The results showed higher recombinant protein expression in the single-promoter vector compared to the dual-promoter vector [84]. However, the FMDV 3C was placed under the p10 promoter, which has been shown to be active a few hours earlier than the polyhedrin promoter and could have led to decreased protein synthesis

due to its deleterious effects on host cells, as discussed previously [113]. An alternate strategy to avoid the use of 3C protease could be to utilize the 2A protease for cleaving the P1 polyprotein into the individual capsid proteins. A study generating FMDV VLPs utilizing only the 2A protease has proven to be successful for VLP formation [100].

Further evaluations need to be performed to determine if the protein band at approximately 55kDa is indeed a VP3-VP1 fusion protein. In addition, performing SVV3C evaluations would give a better idea of SVV3C protein expression inside the cell during infection and its release into the supernatant. The baculovirus system has the potential of creating a SVV VLP, but determining why the viral capsid proteins do not readily form VLPs in baculovirus-infected insect cells requires further investigations.

CHAPTER 4: CONCLUSION

Recent SVV outbreaks have prompted increased interest and research to better understand the virus and its role in SIVD. Although SVV is not a severely debilitating disease, the risk of foreign swine vesicular diseases entering our borders undetected due to unreported SVV clinical signs would have a devastating impact on the swine industry. A vaccine would reduce the occurrence of SVV vesicles and support rapid identification of foreign swine vesicular diseases necessary to maintain biosecurity.

The BEVS has been used to produce several licensed vaccine products for humans and animals. The VLP assembly of many picornavirus proteins has also been proven using the BEVS. The BEVS would be an ideal platform to provide a rapid method for producing a safe and efficacious VLP-based SVV vaccine.

The research performed within this thesis produced SVV capsid proteins detected by specific antibodies in Western blot. This was similar to findings of many other baculovirus-expressed recombinant capsid proteins of picornaviruses [9,64,91,98]. However, the SVV capsid proteins produced did not go on to form a higher ordered VLP structure. The lack of SVV VLP formation could be due to several factors and indicates that further research is warranted to better understand the deficiencies in the SVV VLP assembly process in the BEVS.

Many common viruses of the *Picornaviridae* family such as poliovirus, coxsackievirus A, enteroviruses, swine vesicular disease virus and foot-and-mouth disease virus have proven VLP assembly in the research stage. Although, currently there are no VLP-based licensed vaccines for these viruses demonstrating further research efforts are needed to overcome herein described hurdles. The research conducted for SVV VLP assembly provides increased understanding of the capsid formation of picornaviruses and

differences in efficiency of VLP formation. Even though SVV VLP assembly did not occur, the recombinant capsid proteins expressed could be purified and evaluated as vaccine antigens. Additionally, they provide value as reagents in neutralization assay, ELISA, and serological detection of SVV.

APPENDIX: FIGURES, TABLES & EM IMAGES

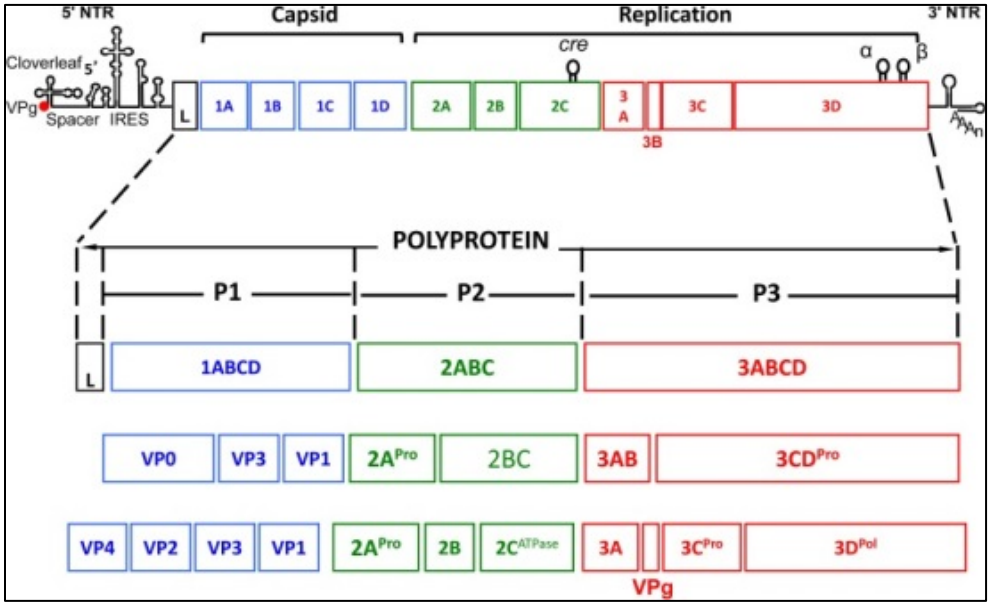


Fig. 1. Genome structure of picornavirus and polyprotein processing [5].

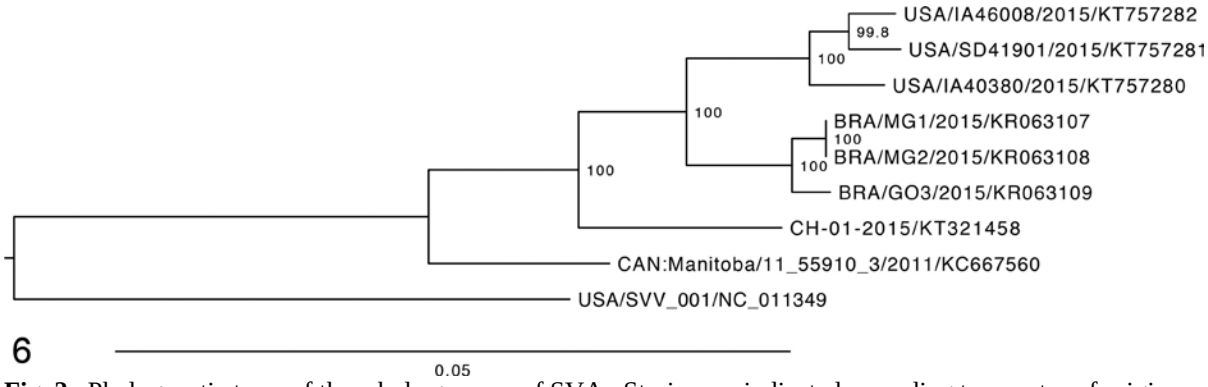


Fig. 2. Phylogenetic trees of the whole genome of SVA. Strains are indicated according to country of origin: Brazil (BRA), Canada (CAN), China (CH), and the USA [11].

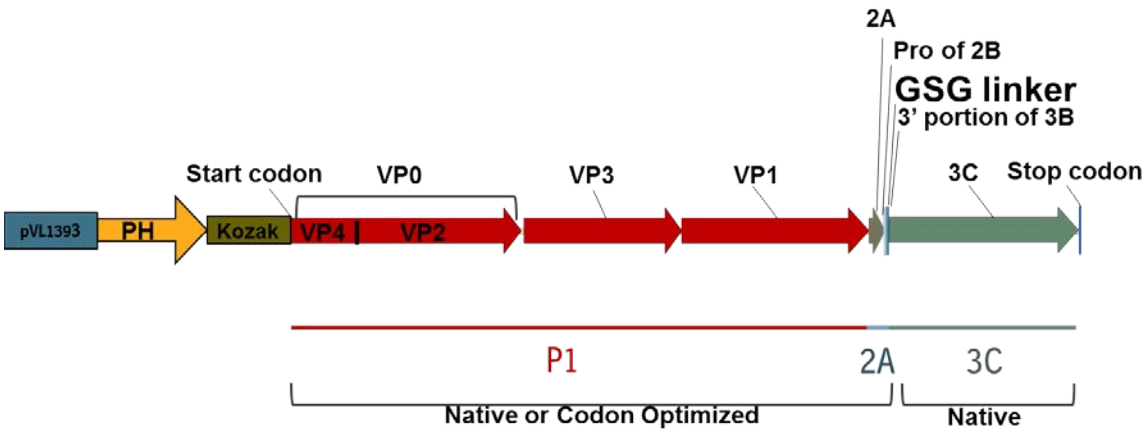


Fig. 3. Seneca Valley virus baculovirus construct designs. SVVP13C and SVVP13C-CO (codon optimized) genes in the pVL1393 transfer vector for the baculovirus expression vector system.

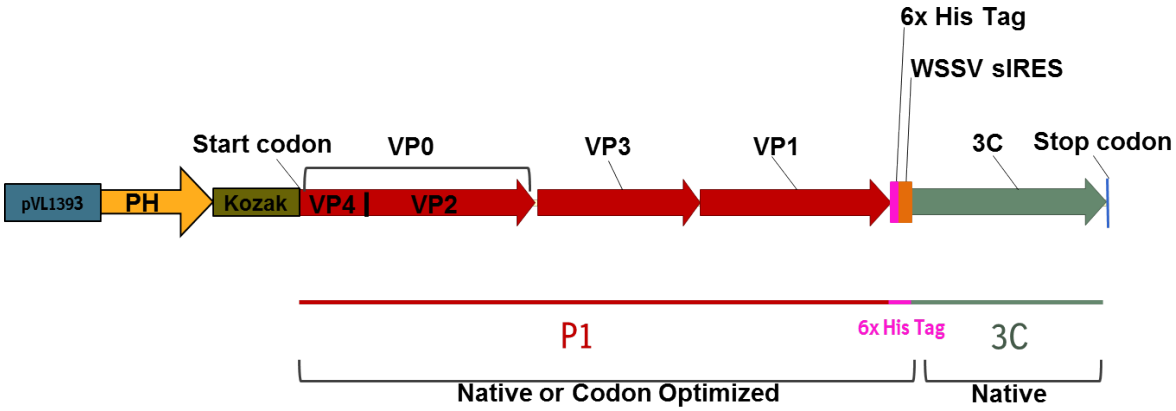


Fig. 4. WSSV sIRES-split his-tagged SVVP1 with SVV3C baculovirus construct designs. SVVP1-His-sIRES-SVV3C and SVVP1CO-His-sIRES-SVV3C (codon optimized) genes in the pVL1393 transfer vector for the baculovirus expression system.

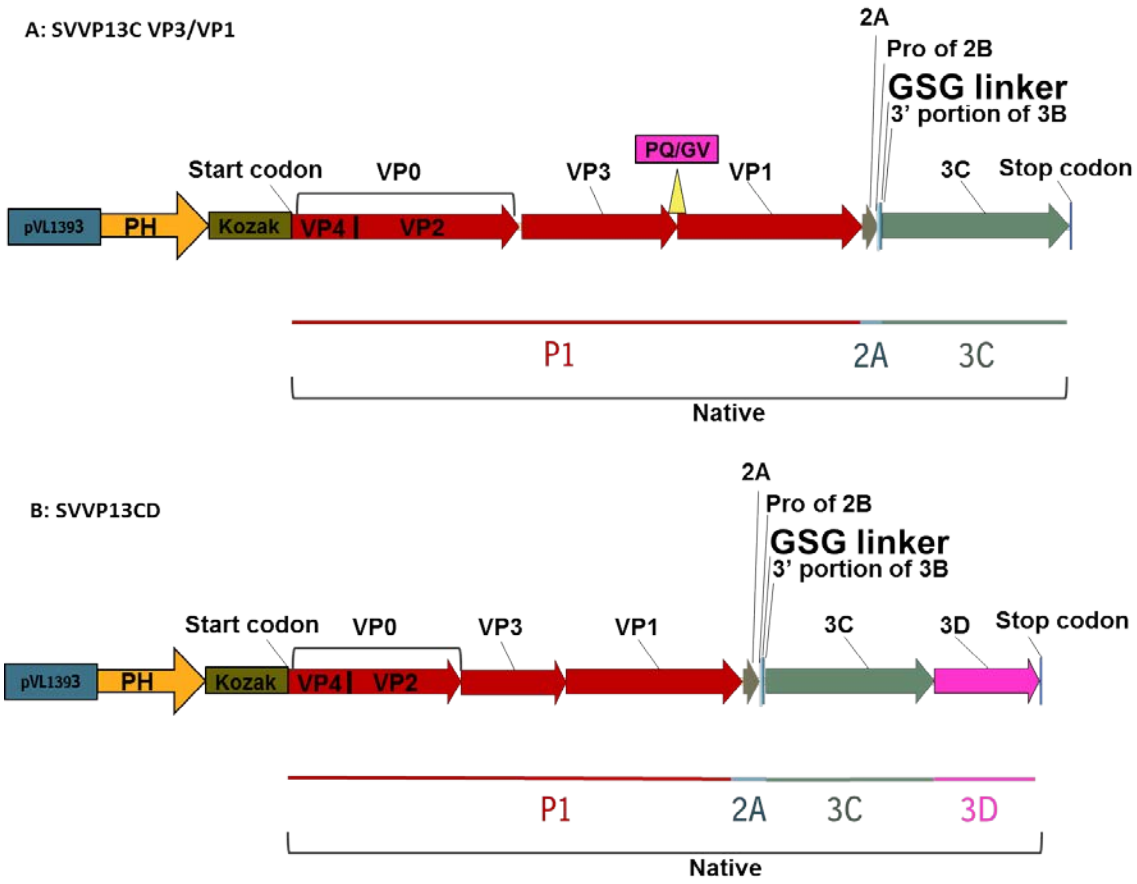


Fig. 5. SVVP13C VP3/VP1 and SVVP13CD baculovirus construct designs. SVVP13C VP3/VP1 construct design (A) to mutate the VP3/VP1 cleavage site and SVVP13CD construct design (B) adding the 3D sequence to the C-terminal end of 3C.

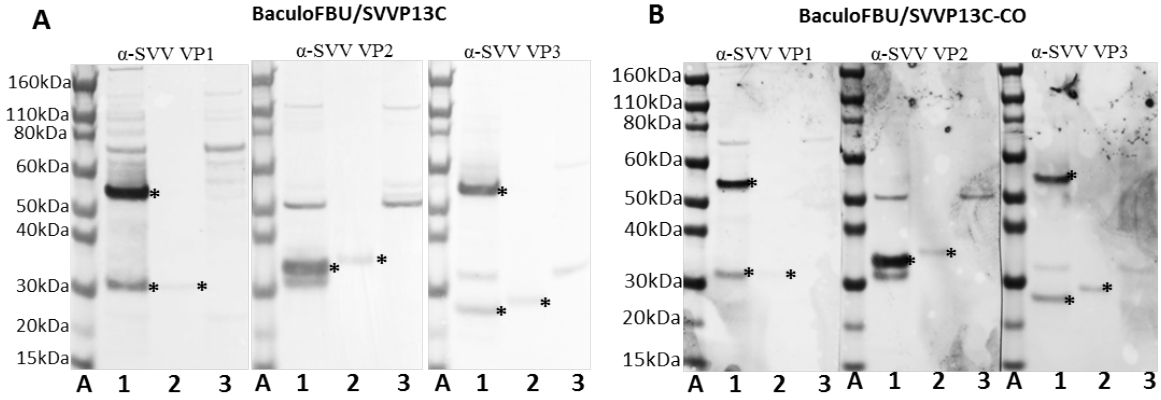


Fig. 6. SVV Recombinant Capsid Protein Expression. Western blots of BaculoFBU/SVVP13C (A) and BaculoFBU/SVVP13C-CO (B) supernatant samples compared to native SVV antigen detected with α-SVV VP1, α-SVV VP2 and α-SVV VP3 cross-adsorbed rabbit polyclonal antibodies. Lane A- Protein Standard, Lane 1-SVV Baculo Harvest Supernatant Lane 2-SVV Antigen and Lane 3-Baculo Harvest Supernatant Neg. Control. Expected Band Sizes: Full Length SVV P1= ~95kDa, VP1 = ~29kDa, VP2= ~32kDA, VP3= ~26kDA

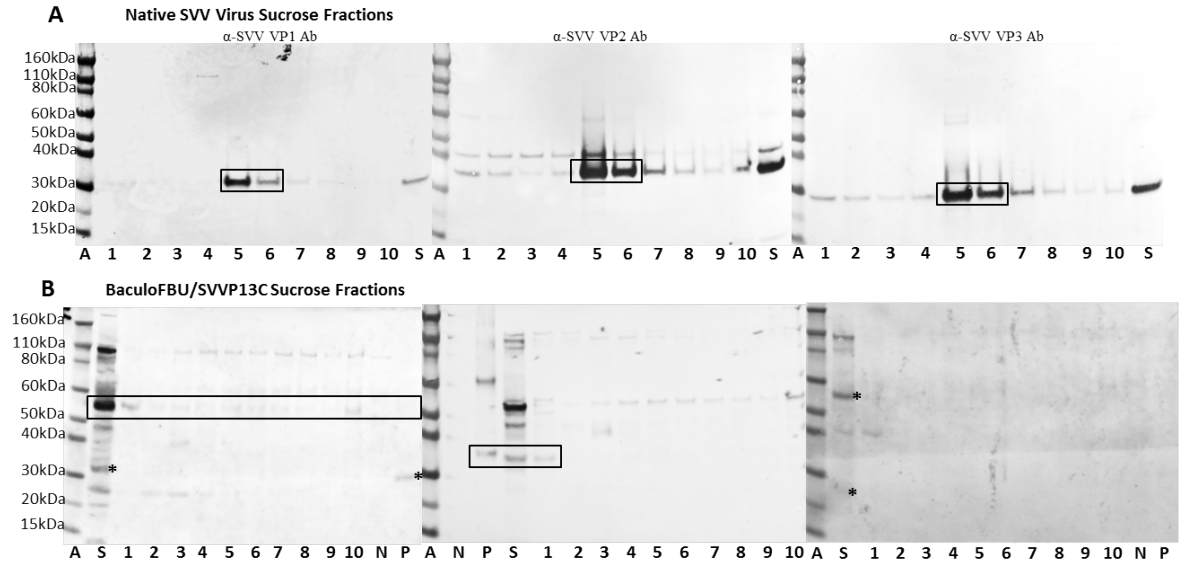


Fig. 7. Western blots of native SVV virus sucrose fractions (A) and BaculoFBU/SVVP13C sucrose fractions (B) with α-SVV VP1, α-SVV VP2 and α-SVV VP3 rabbit polyclonal antibodies. Lane A- Protein Standard, Lanes 1-10- Sucrose fractions 1-10, N- BaculoFBU/No Insert Negative Control, P- Positive Control Native inactivated SVV and S- Starting sample for sucrose gradient.
Expected Band Sizes: Full Length SVV P1= ~95kDa, VP1 = ~29kDa, VP2= ~32kDA, VP3= ~26kDA

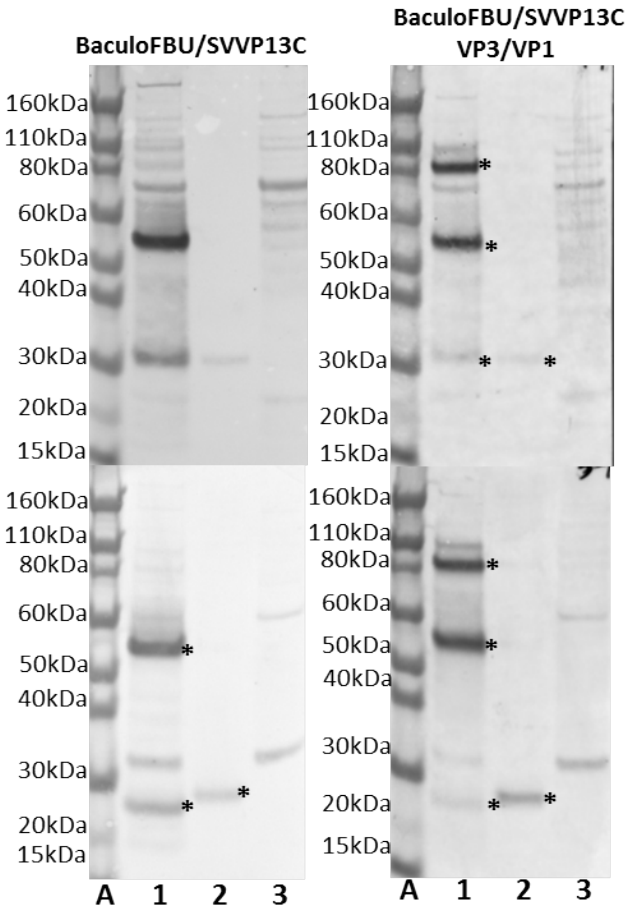


Fig. 8. Western blots of BaculoFBU/SVVP13C and BaculoFBU/SVVP13C VP3/VP1 supernatant harvests with α-SVV VP1 antibody (top row) and α-SVV VP3 (bottom row) rabbit polyclonal antibodies. Lane A-Protein Standard, Lane 1-SVV Baculo Harvest Supernatant, Lane 2-SVV Antigen and Lane 3-Baculo Harvest Supernatant Neg. Control. Expected Band Sizes: Full Length SVV P1= ~95kDa, VP1 = ~29kDa, VP3= ~26kDA, VP3-VP1= ~56kDa

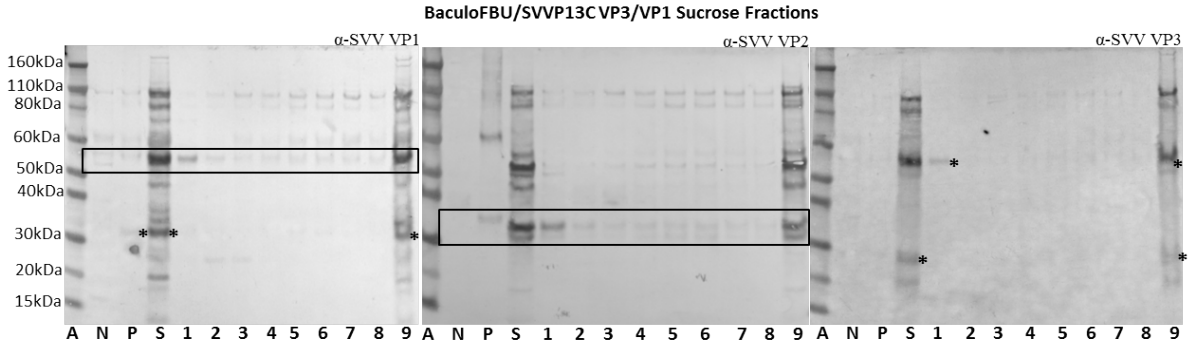


Fig. 9. Western blots of BaculoFBU/SVVP13C VP3/VP1 sucrose fractions with α-SVV VP1, α-SVV VP2 and α-SVV VP3 rabbit polyclonal antibodies. Lane A-Protein Standard, N= BaculoFBU/No Insert Negative Control, P= Positive Control native inactivated SVV, S= SVVP13C VP3/VP1 pellet re-suspended in TBS and Lane 1-9 = Sucrose Fractions 1-9.
Expected Band Sizes: Full Length SVV P1= ~95kDa, VP1 = ~29kDa, VP2= ~32kDa and VP3= ~26kDA

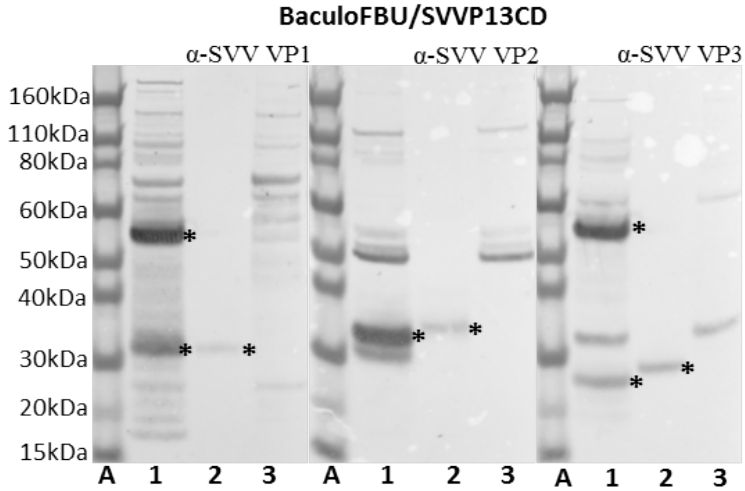


Fig. 10. Western blots of BaculoFBU/SVVP13CD Supernatant Harvest detected with α-SVV VP1, α-SVV VP2 and α-SVV VP3 rabbit polyclonal antibodies. Lane A-Protein Standard, Lane 1-SVV Baculo Harvest Supernatant Lane 2-SVV Antigen and Lane 3-Baculo Harvest Supernatant Neg. Control.
Expected Band Sizes: Full Length SVV P1= ~95kDa, VP1 = ~29kDa, VP2= ~32kDA and VP3= ~26kDA

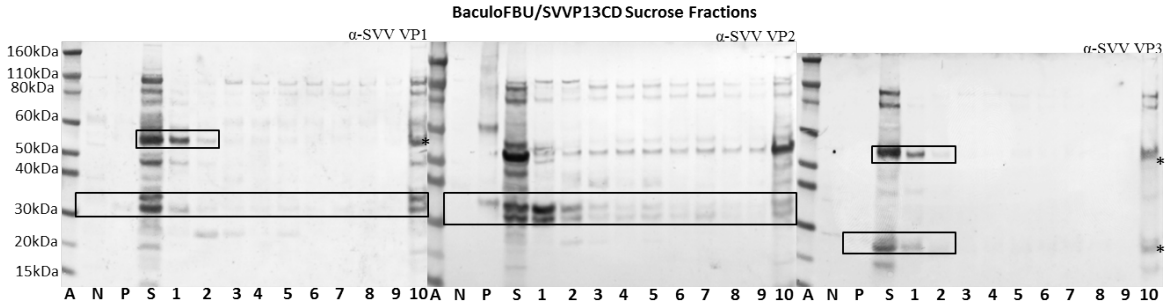


Fig. 11. Western blots of BaculoFBU/SVVP13CD sucrose fractions with α-SVV VP1, α-SVV VP2 and α-SVV VP3 rabbit polyclonal antibodies. Lane A-Protein Standard, N= BaculoFBU/No Insert Negative Control, P= Positive Control Native inactivated SVV, S= SVVP13CD pellet re-suspended in TBS and Lane 1-10 = Sucrose Fractions 1-10.
Expected Band Sizes: Full Length SVV P1= ~95kDa, VP1 = ~29kDa, VP2= ~32kDa and VP3= ~26kDA

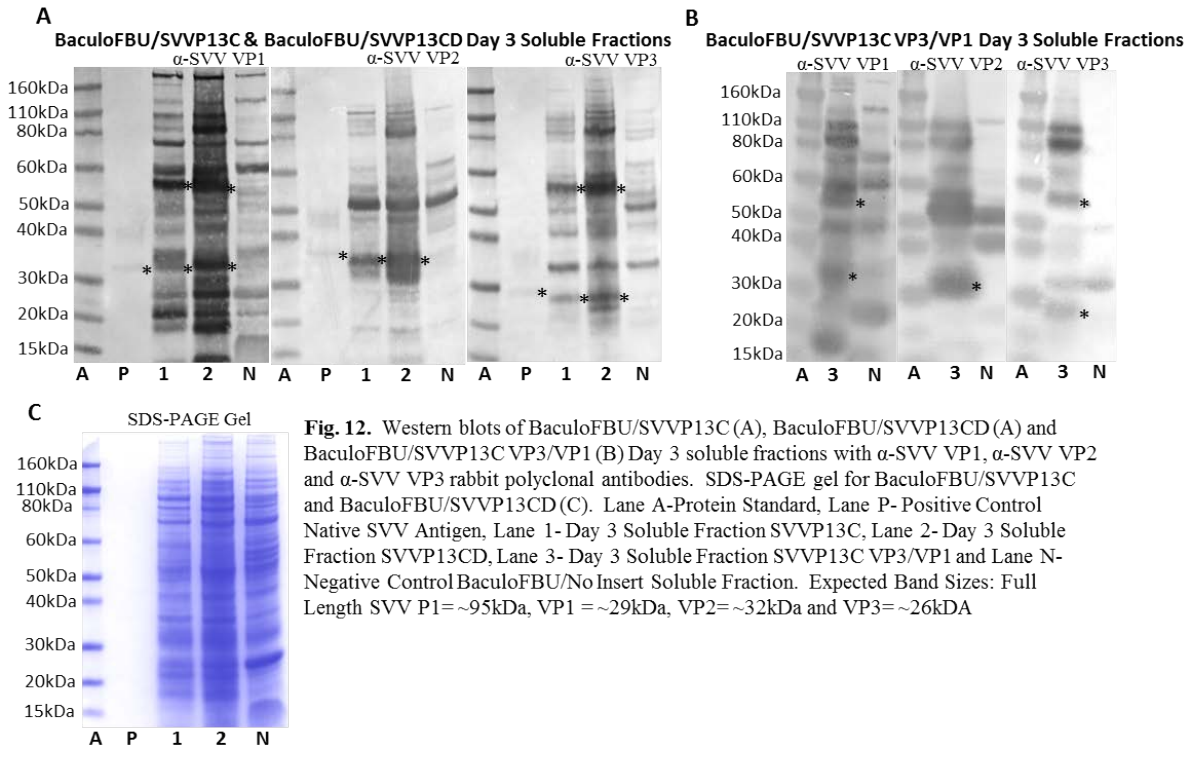


Fig. 12. Western blots of BaculoFBU/SVVP13C (A), BaculoFBU/SVVP13CD (A) and BaculoFBU/SVVP13C VP3/VP1 (B) Day 3 soluble fractions with α-SVV VP1, α-SVV VP2 and α-SVV VP3 rabbit polyclonal antibodies. SDS-PAGE gel for BaculoFBU/SVVP13C and BaculoFBU/SVVP13CD (C). Lane A-Protein Standard, Lane P- Positive Control Native SVV Antigen, Lane 1- Day 3 Soluble Fraction SVVP13C, Lane 2- Day 3 Soluble Fraction SVVP13CD, Lane 3- Day 3 Soluble Fraction SVVP13C VP3/VP1 and Lane N- Negative Control BaculoFBU/No Insert Soluble Fraction. Expected Band Sizes: Full Length SVV P1= ~95kDa, VP1 = ~29kDa, VP2= ~32kDa and VP3= ~26kDA

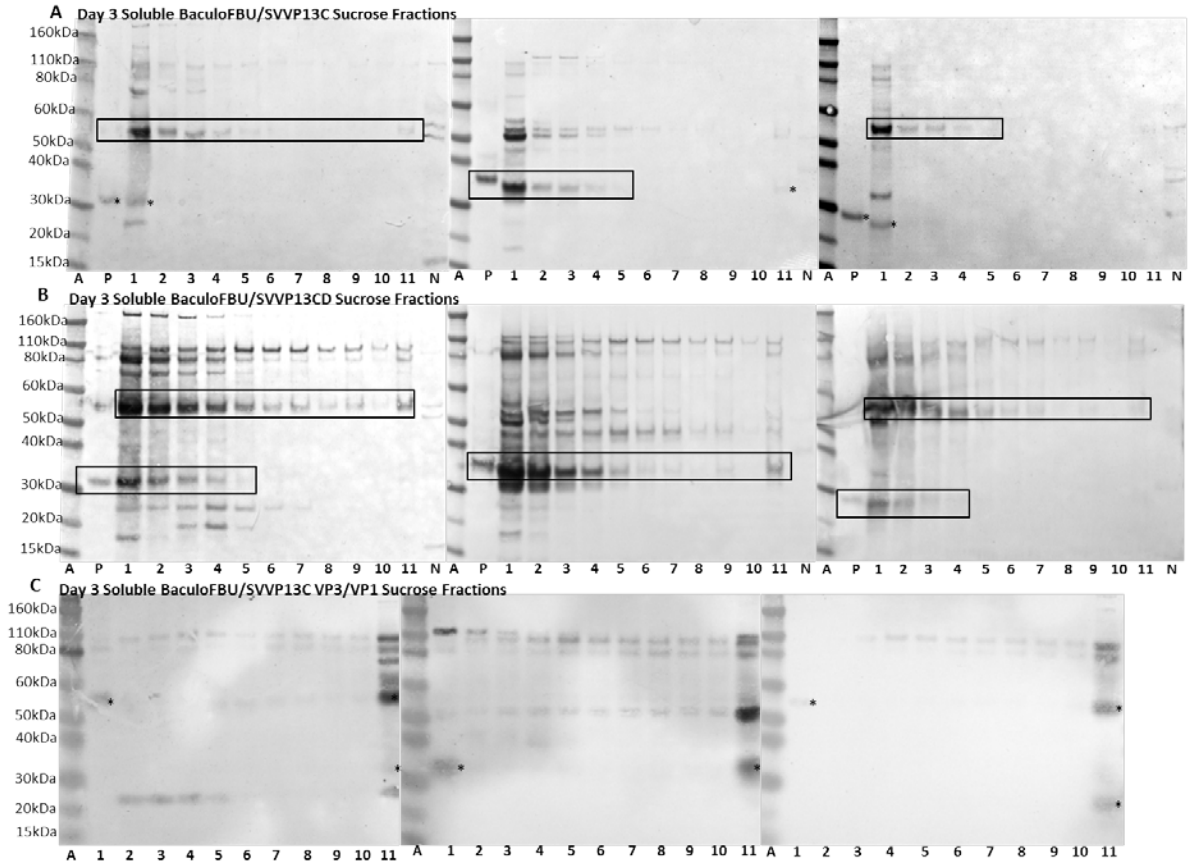


Fig. 13. Western blots of sucrose fractions of Day 3 Soluble BaculoFBU/SVVP13C, BaculoFBU/SVVP13CD and BaculoFBU/SVVP13C VP3/VP1 with α-SVV VP1, α-SVV VP2 and α-SVV VP3 rabbit polyclonal antibodies. Lane A- Protein Standard, Lane B-SVV Baculo Harvest Supernatant Lane C-SVV Antigen and Lane D- Supernatant Baculo Neg. Control. Lanes 1-10- Sucrose Fractions 1-10 and Lane 11- SVVP13C VP3/VP1 Starting Pellet. Expected Band Sizes: Full Length SVV P1= ~95kDa, VP1 = ~29kDa, VP2= ~32kDA, VP3= ~26kDA and SVV VP1+VP3= ~56kDa.

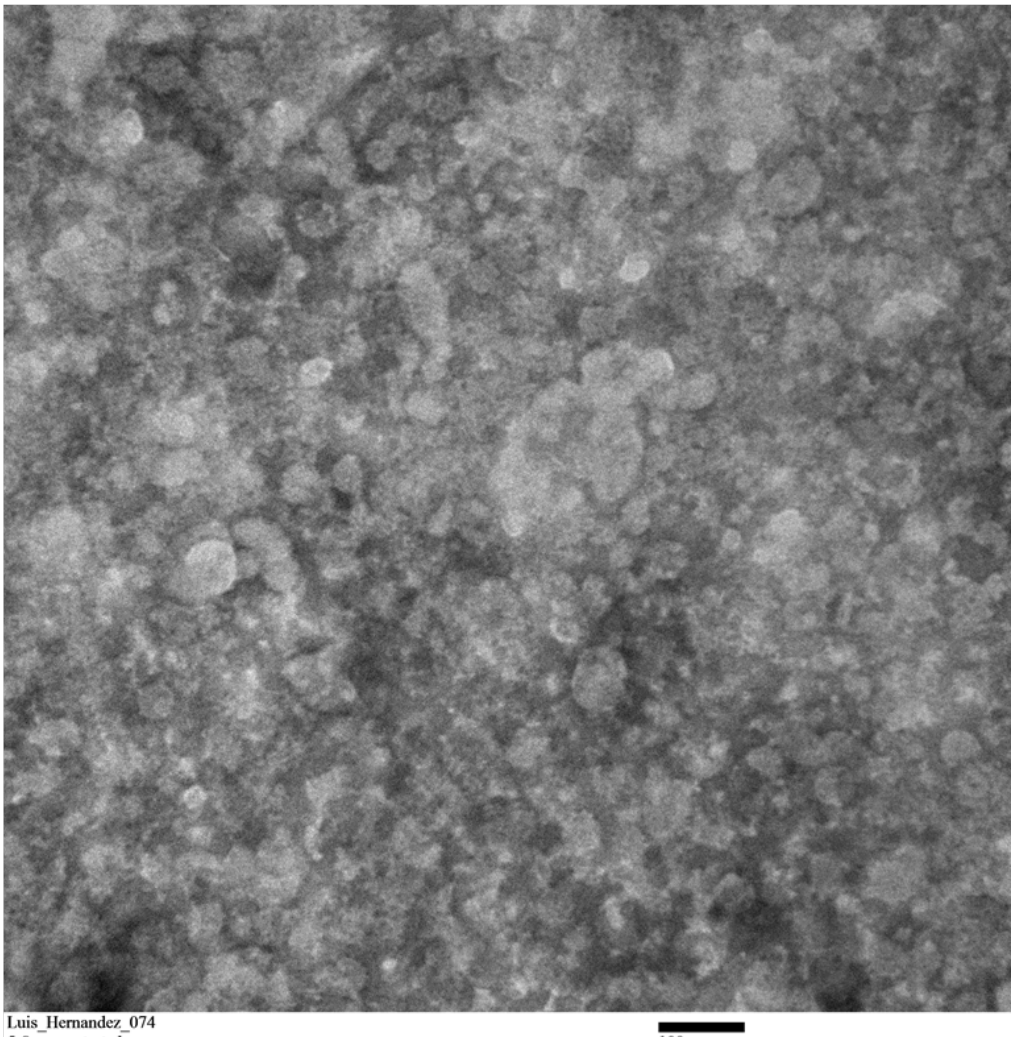
Table 1. Primer Sequences for SVVP1-His-SVV3C and SVVP1CO-His-SVV3C

Primer	Sequence (5' - 3')	Lot #
P3219012A (SVVP1 Fwd)	GGATCCGCCACCATGGGTAATGTTCA	3219-013A
P3219039A (SVVP1 His Rev)	GCGGCCGCTCAGTGGTGGTGGTGGTGGTGTTGCATCA GCATCTTTTGCTTGTAGCTGC	3219-042A
P3219012C (SVVP1-CO Fwd)	GGATCCGCCACCATGGGCAACG	3219-013C
P3219039B (SVVP1-CO His Rev)	GCGGCCGCTCAGTGGTGGTGGTGGTGGTGTTGCATAA GCATCTTCTGTTTATAGCTACGG	3219-042B
P3219012E (SVV3C Fwd)	ACTAGTATGCAGCCCAACGTGGACATGGGCTTT	3219-013E
P3219039C (SVV3C Rev)	GAGCTCTCATTGCATTGTAGCCAGAGGCTCACCGA	3219-042C

Table 2. Primer Sequences for SVVP13C VP3/VP1 and SVVP13CD

Primer	Sequence (5' - 3')	Lot #
P3219165A (VP3/VP1 Fwd)	CTTCCTACGTGCCTCAGGGGGTTGACAACGCCGAGACTGGG	3219-168A
P3219165B (VP3/VP1 Rev)	CCCAGTCTCGGCGTTGTCAACCCCCTGAGGCACGTAGGAAG	3219-168B
P3219166A (SVV3D Fwd)	TACAATGCAAGGACTGATGACTGAGCTAGAGCCTG	3219-171A
P3219166B (SVV3C Rev)	TCAGTCATCAGTCCTTGCATTGTAGCCAGAG	3219-171B
P3219166C (SVV3D Rev)	GCGGCCGCTCAGTCGAACAAGGCCCTCCATCT	3219-171C

EM Image of BaculoFBU/SVVP13CD Supernatant Harvest VLP sample

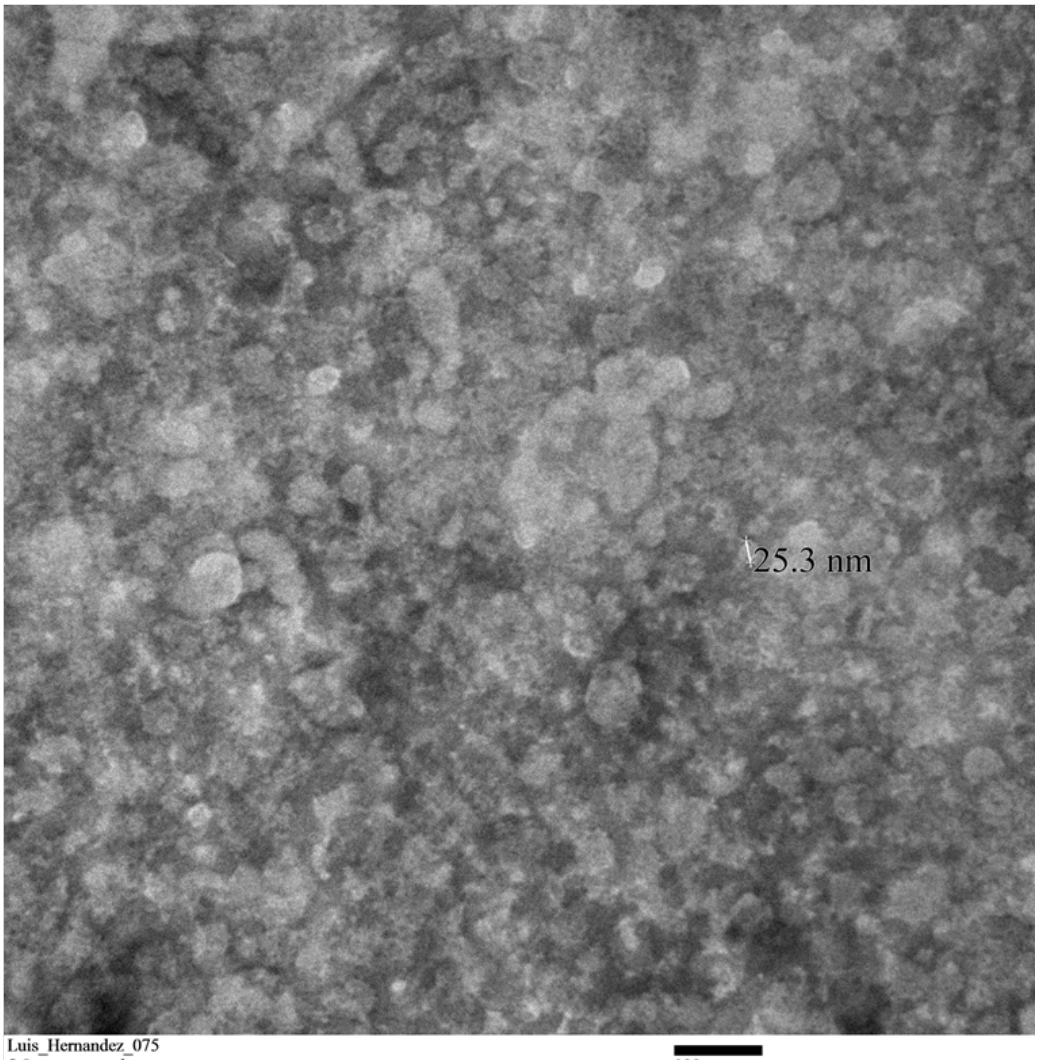


Luis_Hernandez_074
5-8 concentrated
Print Mag: 153000x @ 7.0 in
14:52:45 8/26/2016

Camera: Hamamatsu ORCA HR Camera , Exposure(ms): 1300 Gain: 2, Bin: 1
Gamma: 1.00, No Sharpening, Normal Contrast

100 nm
HV=80.0kV
Direct Mag: 98000x
National Animal Disease Center

EM Image of BaculoFBU/SVVP13CD Supernatant Harvest VLP sample (with measurements)

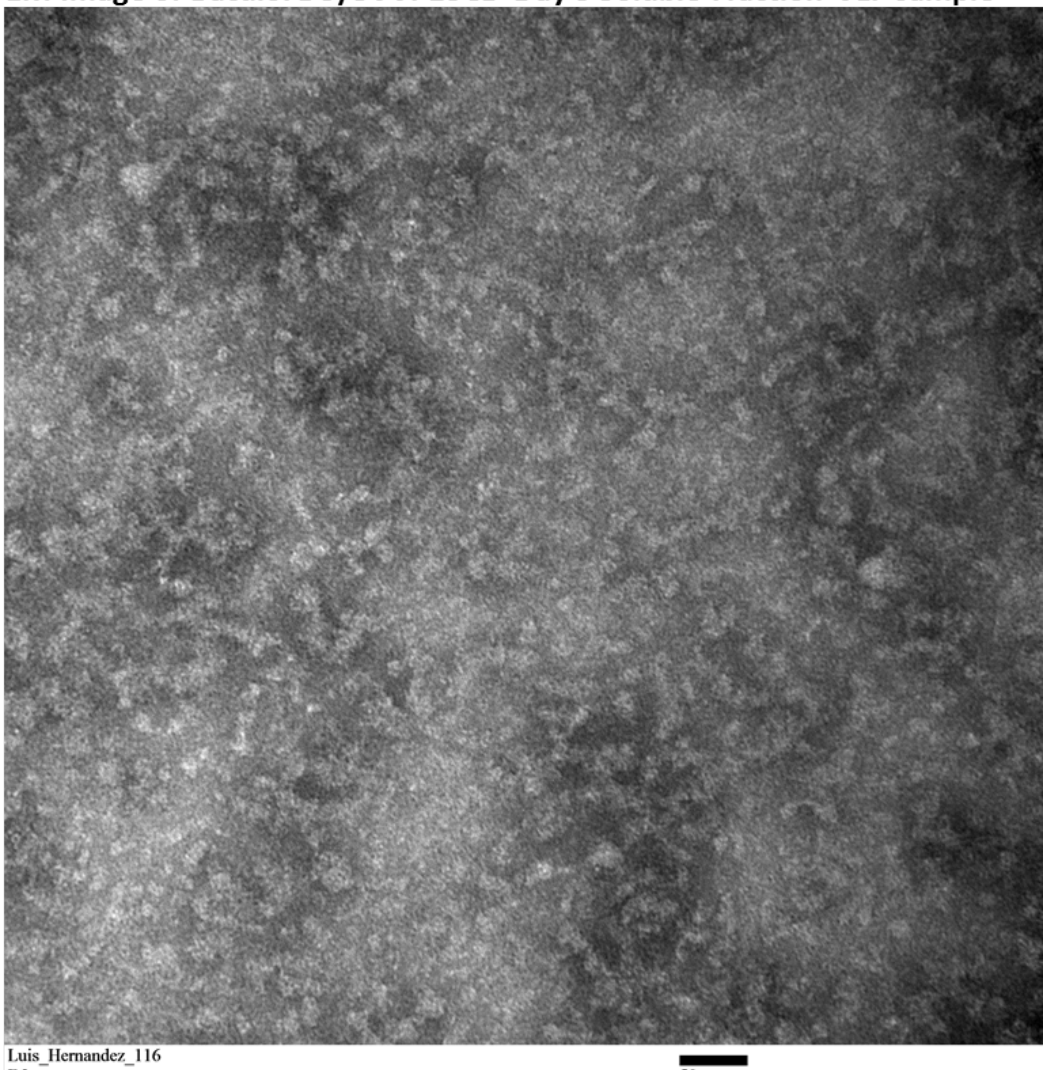


Luis_Hernandez_075
5-8 concentrated
Print Mag: 153000x @ 7.0 in
14:52:45 8/26/2016

Camera: Hamamatsu ORCA HR Camera , Exposure(ms): 1300 Gain: 2, Bin: 1
Gamma: 1.00, No Sharpening, Normal Contrast

100 nm
HV=80.0kV
Direct Mag: 98000x
National Animal Disease Center

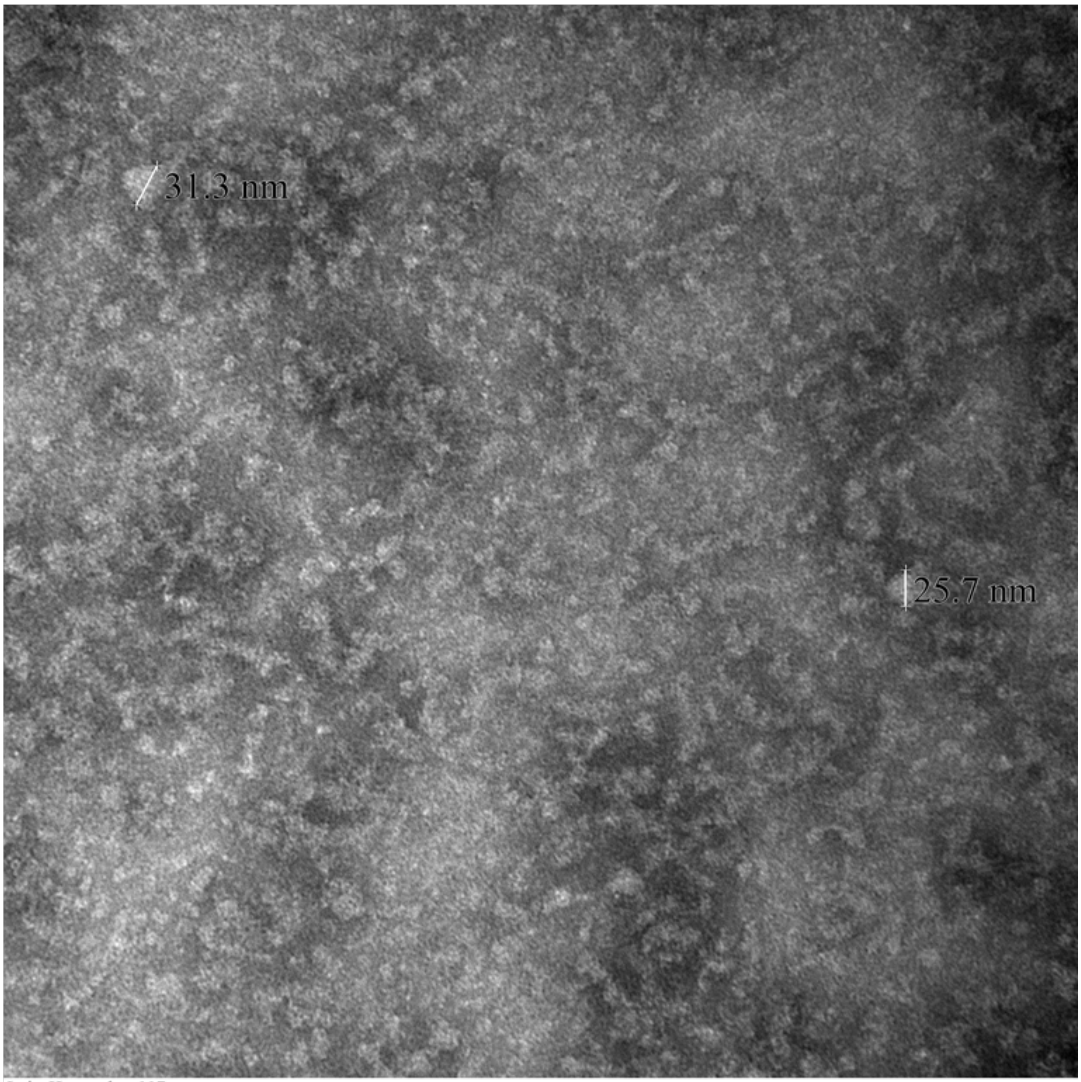
EM Image of BaculoFBU/SVVP13CD Day 3 Soluble Fraction VLP sample



Luis_Hernandez_116
D3
2% PTA 3'
Print Mag: 234000x @ 7.0 in
13:49:46 6/16/2017
Camera: Hamamatsu ORCA HR Camera , Exposure(ms): 1300 Gain: 2, Bin: 1
Gamma: 1.00, No Sharpening, Normal Contrast

50 nm
HV=80.0kV
Direct Mag: 150000x
National Animal Disease Center

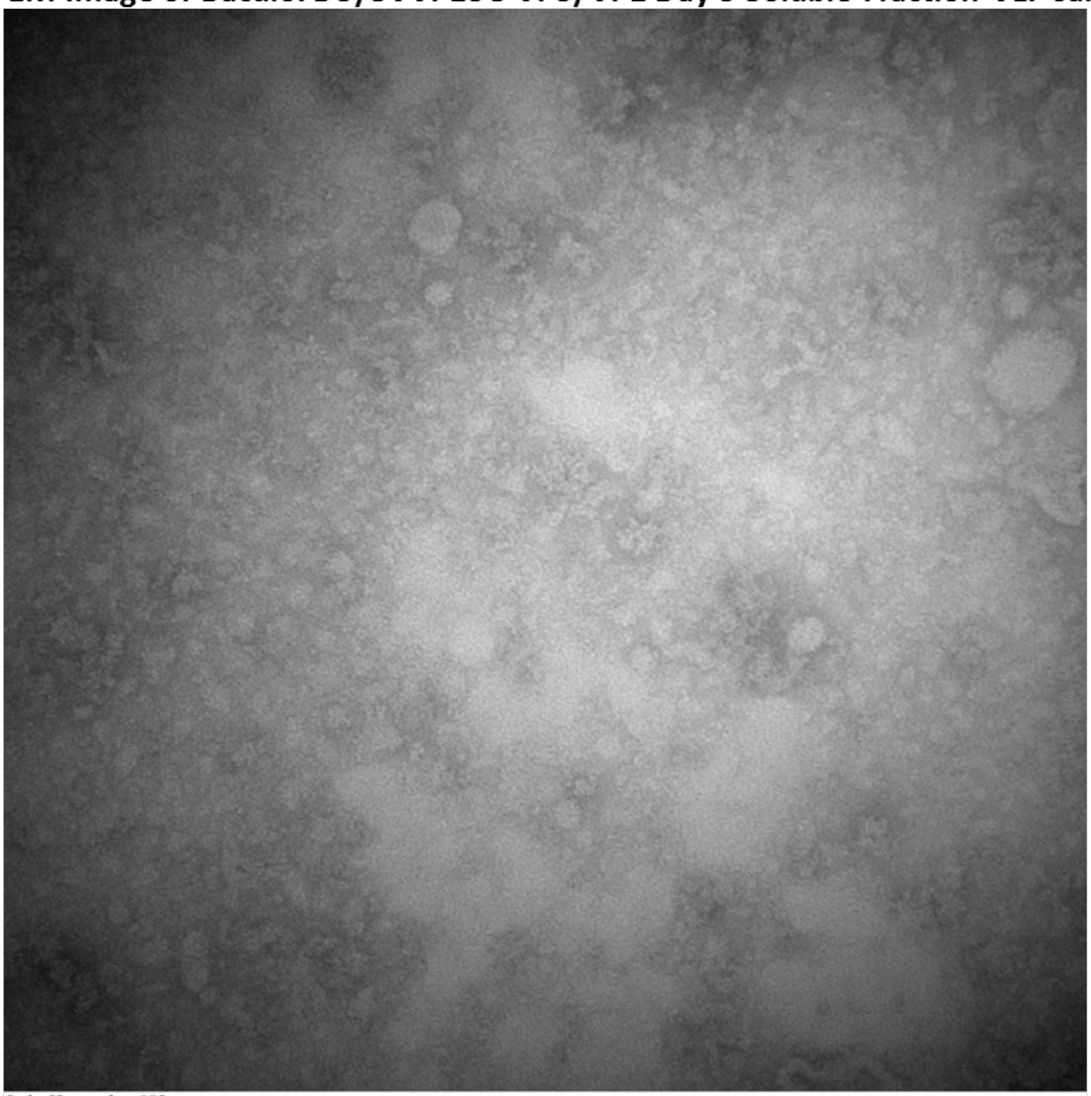
EM Image of BaculoFBU/SVVP13CD Day 3 Soluble Fraction VLP sample (with measurements)



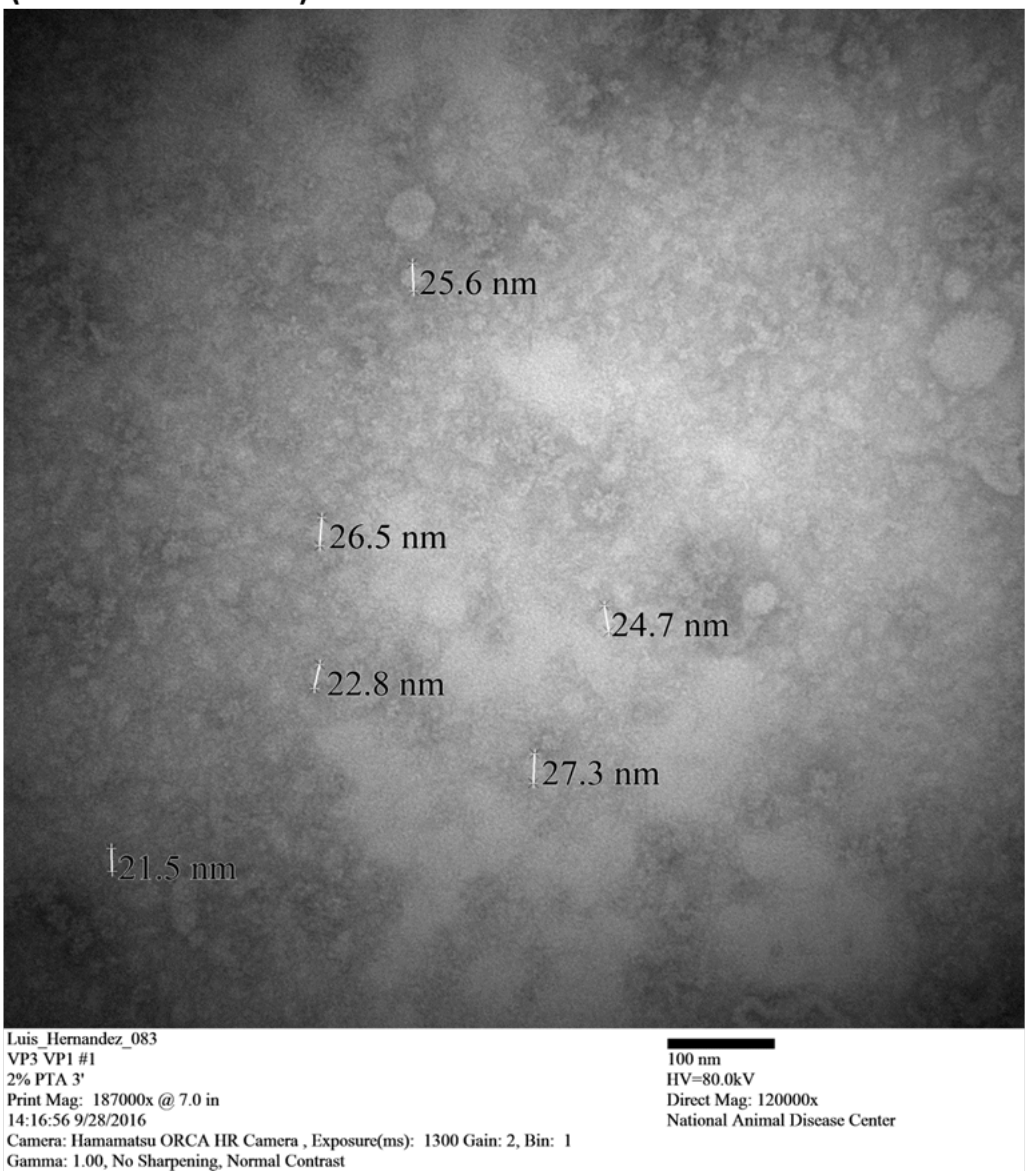
Luis_Hernandez_117
D3
2% PTA 3'
Print Mag: 234000x @ 7.0 in
13:49:46 6/16/2017
Camera: Hamamatsu ORCA HR Camera , Exposure(ms): 1300 Gain: 2, Bin: 1
Gamma: 1.00, No Sharpening, Normal Contrast

50 nm
HV=80.0kV
Direct Mag: 150000x
National Animal Disease Center

EM Image of BaculoFBU/SVVP13C VP3/VP1 Day 3 Soluble Fraction VLP sample



EM Image of BaculoFBU/SVVP13C VP3/VP1 Day 3 Soluble Fraction VLP sample (with measurements)



REFERENCES

1. King A, Lefkowits E, Adams MJ, et al. Virus Taxonomy. *Ninth Report of the International Committee on Taxonomy of Viruses* 2011:872-873.

2. Zell R, Delwart E, Gorbalenya AE, et al. ICTV Virus Taxonomy Profile: Picornaviridae. *J Gen Virol* 2017;98:2421-2422.

3. Strauss J, Strauss E. Viruses and Human Diseases. 2008;2nd Edition:63.

4. Stanway G. Structure, function and evolution of picornaviruses. *J Gen Virol* 1990;71 (Pt 11):2483-2501.

5. Jiang P, Liu Y, Ma HC, et al. Picornavirus morphogenesis. *Microbiol Mol Biol Rev* 2014;78:418-437.

6. Hales LM, Knowles NJ, Reddy PS, et al. Complete genome sequence analysis of Seneca Valley virus-001, a novel oncolytic picornavirus. *J Gen Virol* 2008;89:1265-1275.

7. Leme RA, Alfieri AF, Alfieri AA. Update on Senecavirus Infection in Pigs. *Viruses* 2017;9.

8. Somasundaram B, Lua L. Development of picornavirus-like particle vaccines. *Pharmaceutical BioProcessing* 2015;3: 45–59.

9. Chung YC, Huang JH, Lai CW, et al. Expression, purification and characterization of enterovirus-71 virus-like particles. *World J Gastroenterol* 2006;12:921-927.

10. Wu Q, Zhao X, Chen Y, et al. Complete Genome Sequence of Seneca Valley Virus CH-01-2015 Identified in China. *Genome Announc* 2016;4.

11. Segales J, Barcellos D, Alfieri A, et al. Senecavirus A. *Vet Pathol* 2017;54:11-21.

12. Knowles NJ, Hales LM, Jones BH, et al. Epidemiology of Seneca Valley virus: identification and characterization of isolates from pigs in the United States.pdf>. *Northern Lights EUROPIC 2006: XIVth Meeting of the European Study Group on Molecular Biology of Picornaviruses* 2006;Saariselkä, Inari, Finland.

13. Venkataraman S, Reddy SP, Loo J, et al. Crystallization and preliminary X-ray diffraction studies of Seneca Valley virus-001, a new member of the Picornaviridae family. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 2008;64:293-296.

14. Gorbalenya AE, Blinov VM, Donchenko AP. Poliovirus-encoded proteinase 3C: a possible evolutionary link between cellular serine and cysteine proteinase families. *FEBS Lett* 1986;194:253-257.

15. Bazan JF, Fletterick RJ. Viral cysteine proteases are homologous to the trypsin-like family of serine proteases: structural and functional implications. *Proc Natl Acad Sci U S A* 1988;85:7872-7876.

16. Allaire M, Chernaia MM, Malcolm BA, et al. Picornaviral 3C cysteine proteinases have a fold similar to chymotrypsin-like serine proteinases. *Nature* 1994;369:72-76.

17. Leedom Larson K, Lambert T, Killoran K. Seneca Valley Virus. Swine Health Information Center and Center for Food Security and Public Health 2015.

18. Corner S, Singh K. Seneca Valley Virus and Vesicular Lesions in a Pig with Idiopathic Vesicular Disease. *Journal of Veterinary Science & Technology* 2012;03.

19. Gimenez-Lirola LG, Rademacher C, Linhares D, et al. Serological and Molecular Detection of Senecavirus A Associated with an Outbreak of Swine Idiopathic Vesicular Disease and Neonatal Mortality. *J Clin Microbiol* 2016;54:2082-2089.

20. Canning P, Canon A, Bates JL, et al. Neonatal Mortality, Vesicular Lesions and Lameness Associated with Senecavirus A in a U.S. Sow Farm. *Transbound Emerg Dis* 2016;63:373-378.

21. Vannucci FA, Linhares DC, Barcellos DE, et al. Identification and Complete Genome of Seneca Valley Virus in Vesicular Fluid and Sera of Pigs Affected with Idiopathic Vesicular Disease, Brazil. *Transbound Emerg Dis* 2015;62:589-593.

22. Pasma T, Davidson S, Shaw SL. Idiopathic vesicular disease in swine in Manitoba. *Can Vet J* 2008;49:84-85.

23. Chen Z, Guo X, Ge X, et al. Protective immune response in mice vaccinated with a recombinant adenovirus containing capsid precursor polypeptide P1, nonstructural

protein 2A and 3C protease genes (P12A3C) of encephalomyocarditis virus. *Vaccine* 2008;26:573-580.

24. Montiel N, Buckley A, Guo B, et al. Vesicular Disease in 9-Week-Old Pigs Experimentally Infected with Senecavirus A. *Emerg Infect Dis* 2016;22:1246-1248.

25. Buckley A, Kulshreshtha V, van Geelen A, et al. Senecavirus A: Overview of experimental studies. *48th Annual Meeting of the American Association of Swine Veterinarians* 2017.

26. Sturos M, Murray D, Reicks D, et al. Natural and experimentally-induced Senecavirus A infections in boars. *Swine Health Information Center* 2017;United States Department of Agriculture.

27. Reddy PS, Burroughs KD, Hales LM, et al. Seneca Valley virus, a systemically deliverable oncolytic picornavirus, and the treatment of neuroendocrine cancers. *J Natl Cancer Inst* 2007;99:1623-1633.

28. Miles LA, Burga LN, Gardner EE, et al. Anthrax toxin receptor 1 is the cellular receptor for Seneca Valley virus. *J Clin Invest* 2017;127:2957-2967.

29. Poirier JT, Reddy PS, Idamakanti N, et al. Characterization of a full-length infectious cDNA clone and a GFP reporter derivative of the oncolytic picornavirus SVV-001. *J Gen Virol* 2012;93:2606-2613.

30. Morton CL, Houghton PJ, Kolb EA, et al. Initial testing of the replication competent Seneca Valley virus (NTX-010) by the pediatric preclinical testing program. *Pediatr Blood Cancer* 2010;55:295-303.

31. Poirier JT, Dobromilskaya I, Moriarty WF, et al. Selective tropism of Seneca Valley virus for variant subtype small cell lung cancer. *J Natl Cancer Inst* 2013;105:1059-1065.

32. Wadhwa L, Hurwitz MY, Chevez-Barrios P, et al. Treatment of invasive retinoblastoma in a murine model using an oncolytic picornavirus. *Cancer Res* 2007;67:10653-10656.

33. Yu L, Baxter PA, Zhao X, et al. A single intravenous injection of oncolytic picornavirus SVV-001 eliminates medulloblastomas in primary tumor-based orthotopic xenograft mouse models. *Neuro Oncol* 2011;13:14-27.

34. Rudin CM, Poirier JT, Senzer NN, et al. Phase I clinical study of Seneca Valley Virus (SVV-001), a replication-competent picornavirus, in advanced solid tumors with neuroendocrine features. *Clin Cancer Res* 2011;17:888-895.

35. Burke MJ, Ahern C, Weigel BJ, et al. Phase I trial of Seneca Valley Virus (NTX-010) in children with relapsed/refractory solid tumors: a report of the Children's Oncology Group. *Pediatr Blood Cancer* 2015;62:743-750.

36. Report of the committee on transmissible diseases of siwne, 2012.

37. Snelson H. AASV Swine Health Committee Considers SVV. 2015.

38. Saeng-Chuto K, Rodtian P, Temeeyasen G, et al. The first detection of Senecavirus A in pigs in Thailand, 2016. *Transbound Emerg Dis* 2017.

39. Zhang J, Pineyro P, Chen Q, et al. Full-Length Genome Sequences of Senecavirus A from Recent Idiopathic Vesicular Disease Outbreaks in U.S. Swine. *Genome Announc* 2015;3.

40. Report of the committee on transmissible diseases of siwne. 2015.

41. Diagnosticians AAoVL. Senecavirus A vesicular disease outbreak in Midwest show and commercial pigs. 2015.

42. Kleid DG, Yansura D, Small B, et al. Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. *Science* 1981;214:1125-1129.

43. Bachrach HL, Moore DM, McKercher PD, et al. Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. *J Immunol* 1975;115:1636-1641.

44. Ye X, Ku Z, Liu Q, et al. Chimeric virus-like particle vaccines displaying conserved enterovirus 71 epitopes elicit protective neutralizing antibodies in mice through divergent mechanisms. *J Virol* 2014;88:72-81.

45. Rademacher C. Case reports from Senecavirus A/Seneca Valley Virus – field experiences in Iowa. Allen D Leman Swine Conference 2015.

46. Sturos M, Vannucci FA. Senecavirus A is still with us. *National Hog Farmer* 2017.

47. Riedel S. Edward Jenner and the history of smallpox and vaccination. *Proc (Bayl Univ Med Cent)* 2005;18:21-25.

48. Kushnir N, Streatfield SJ, Yusibov V. Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. *Vaccine* 2012;31:58-83.

49. Plotkin S. History of vaccination. *Proc Natl Acad Sci U S A* 2014;111:12283-12287.

50. Desmettre P. Veterinary Vaccines in the Development of Vaccination and Vaccinology. *History of Vaccine Development* 2011:329-338.

51. Seo HS. Application of radiation technology in vaccines development. *Clin Exp Vaccine Res* 2015;4:145-158.

52. Alhaj MS. Safety and efficacy profile of commercial veterinary vaccines against Rift Valley Fever disease. *International Journal of Vaccines and Immune System* 2016;1:1-6.

53. Baxter D. Active and passive immunity, vaccine types, excipients and licensing. *Occup Med (Lond)* 2007;57:552-556.

54. Tlaxca JL, Ellis S, Remmele RL, Jr. Live attenuated and inactivated viral vaccine formulation and nasal delivery: potential and challenges. *Adv Drug Deliv Rev* 2015;93:56-78.

55. Brun A, Barcena J, Blanco E, et al. Current strategies for subunit and genetic viral veterinary vaccine development. *Virus Res* 2011;157:1-12.

56. Jeoung HY, Lee WH, Jeong W, et al. Immune responses and expression of the virus-like particle antigen of the porcine encephalomyocarditis virus. *Res Vet Sci* 2010;89:295-300.

57. Roldao A, Mellado MC, Castilho LR, et al. Virus-like particles in vaccine development. *Expert Rev Vaccines* 2010;9:1149-1176.

58. Plummer EM, Manchester M. Viral nanoparticles and virus-like particles: platforms for contemporary vaccine design. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2011;3:174-196.

59. Casal JI. Use of the baculovirus expression system for the generation of virus-like particles. *Biotechnol Genet Eng Rev* 2001;18:73-87.

60. Dong H, Guo HC, Sun SQ. Virus-like particles in picornavirus vaccine development. *Appl Microbiol Biotechnol* 2014;98:4321-4329.

61. Tan M, Jiang X. Subviral particle as vaccine and vaccine platform. *Curr Opin Virol* 2014;6:24-33.

62. Gong M, Zhu H, Zhou J, et al. Cryo-electron microscopy study of insect cell-expressed enterovirus 71 and coxsackievirus a16 virus-like particles provides a structural basis for vaccine development. *J Virol* 2014;88:6444-6452.

63. Guo HC, Sun SQ, Jin Y, et al. Foot-and-mouth disease virus-like particles produced by a SUMO fusion protein system in Escherichia coli induce potent protective immune responses in guinea pigs, swine and cattle. *Vet Res* 2013;44:48.

64. Chung YC, Ho MS, Wu JC, et al. Immunization with virus-like particles of enterovirus 71 elicits potent immune responses and protects mice against lethal challenge. *Vaccine* 2008;26:1855-1862.

65. Cao Y, Lu Z, Sun J, et al. Synthesis of empty capsid-like particles of Asia I foot-and-mouth disease virus in insect cells and their immunogenicity in guinea pigs. *Vet Microbiol* 2009;137:10-17.

66. Rosen E, Stapleton JT, McLinden J. Synthesis of immunogenic hepatitis A virus particles by recombinant baculoviruses. *Vaccine* 1993;11:706-712.

67. Fernandes F, Teixeira AP, Carinhas N, et al. Insect cells as a production platform of complex virus-like particles. *Expert Rev Vaccines* 2013;12:225-236.

68. Lewis SA, Morgan DO, Grubman MJ. Expression, processing, and assembly of foot-and-mouth disease virus capsid structures in heterologous systems: induction of a neutralizing antibody response in guinea pigs. *J Virol* 1991;65:6572-6580.

69. Lee CD, Yan YP, Liang SM, et al. Production of FMDV virus-like particles by a SUMO fusion protein approach in Escherichia coli. *J Biomed Sci* 2009;16:69.

70. Rombaut B, Jore JP. Immunogenic, non-infectious polio subviral particles synthesized in Saccharomyces cerevisiae. *J Gen Virol* 1997;78 (Pt 8):1829-1832.

71. Li HY, Han JF, Qin CF, et al. Virus-like particles for enterovirus 71 produced from Saccharomyces cerevisiae potently elicits protective immune responses in mice. *Vaccine* 2013;31:3281-3287.

72. Zhang C, Ku Z, Liu Q, et al. High-yield production of recombinant virus-like particles of enterovirus 71 in Pichia pastoris and their protective efficacy against oral viral challenge in mice. *Vaccine* 2015;33:2335-2341.

73. Gullberg M, Muszynski B, Organtini LJ, et al. Assembly and characterization of foot-and-mouth disease virus empty capsid particles expressed within mammalian cells. *J Gen Virol* 2013;94:1769-1779.

74. Mignaqui AC, Ruiz V, Perret S, et al. Transient gene expression in serum-free suspension-growing mammalian cells for the production of foot-and-mouth disease virus empty capsids. *PLoS One* 2013;8:e72800.

75. Dalsgaard K, Uttenthal A, Jones TD, et al. Plant-derived vaccine protects target animals against a viral disease. *Nat Biotechnol* 1997;15:248-252.

76. Wigdorovitz A, Perez Filgueira DM, Robertson N, et al. Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. *Virology* 1999;264:85-91.

77. Baculovirus Expression Vector System. Pharmingen 1999;6.

78. Contreras-Gomez A, Sanchez-Miron A, Garcia-Camacho F, et al. Protein production using the baculovirus-insect cell expression system. *Biotechnol Prog* 2014;30:1-18.

79. van Oers MM, Pijlman GP, Vlak JM. Thirty years of baculovirus-insect cell protein expression: from dark horse to mainstream technology. *J Gen Virol* 2015;96:6-23.

80. flashBAC one-step baculovirus protein expression. GenWay BioTech, Inc.

81. Mena JA, Kamen AA. Insect cell technology is a versatile and robust vaccine manufacturing platform. *Expert Rev Vaccines* 2011;10:1063-1081.

82. Kitts PA, Ayres MD, Possee RD. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Res* 1990;18:5667-5672.

83. Fraser MJ. Expression of Eucaryotic Genes in Insect Cell Cultures. *In Vitro Cellular and Developmental Biology* 1989;25:225-235.

84. Ruiz V, Mignaqui AC, Nunez MC, et al. Comparison of strategies for the production of FMDV empty capsids using the baculovirus vector system. *Mol Biotechnol* 2014;56:963-970.

85. Kidd IM, Emery VC. The use of baculoviruses as expression vectors. *Appl Biochem Biotechnol* 1993;42:137-159.

86. Rweyemamu MM, Terry G, Pay TW. Stability and immunogenicity of empty particles of foot-and-mouth disease virus. *Arch Virol* 1979;59:69-79.

87. Rowlands DJ, Sangar DV, Brown F. A comparative chemical and serological study of the full and empty particles of foot-and mouth disease virus. *J Gen Virol* 1975;26:227-238.

88. Tuthill TJ, Harlos K, Walter TS, et al. Equine rhinitis A virus and its low pH empty particle: clues towards an aphthovirus entry mechanism? *PLoS Pathog* 2009;5:e1000620.

89. Curry S, Fry E, Blakemore W, et al. Dissecting the roles of VP0 cleavage and RNA packaging in picornavirus capsid stabilization: the structure of empty capsids of foot-and-mouth disease virus. *J Virol* 1997;71:9743-9752.

90. Grubman MJ, Morgan DO, Kendall J, et al. Capsid intermediates assembled in a foot-and-mouth disease virus genome RNA-programmed cell-free translation system and in infected cells. *J Virol* 1985;56:120-126.

91. Urakawa T, Ferguson M, Minor PD, et al. Synthesis of immunogenic, but non-infectious, poliovirus particles in insect cells by a baculovirus expression vector. *J Gen Virol* 1989;70 (Pt 6):1453-1463.

92. Roosien J, Belsham GJ, Ryan MD, et al. Synthesis of foot-and-mouth disease virus capsid proteins in insect cells using baculovirus expression vectors. *J Gen Virol* 1990;71 (Pt 8):1703-1711.

93. Shen C, Ku Z, Zhou Y, et al. Virus-like particle-based vaccine against coxsackievirus A6 protects mice against lethal infections. *Vaccine* 2016;34:4025-4031.

94. Lin YL, Yu CI, Hu YC, et al. Enterovirus type 71 neutralizing antibodies in the serum of macaque monkeys immunized with EV71 virus-like particles. *Vaccine* 2012;30:1305-1312.

95. Liu Q, Yan K, Feng Y, et al. A virus-like particle vaccine for coxsackievirus A16 potently elicits neutralizing antibodies that protect mice against lethal challenge. *Vaccine* 2012;30:6642-6648.

96. Li Z, Yi Y, Yin X, et al. Expression of foot-and-mouth disease virus capsid proteins in silkworm-baculovirus expression system and its utilization as a subunit vaccine. *PLoS One* 2008;3:e2273.

97. Li Z, Yi Y, Yin X, et al. Development of a foot-and-mouth disease virus serotype A empty capsid subunit vaccine using silkworm (Bombyx mori) pupae. *PLoS One* 2012;7:e43849.

98. Hu YC, Hsu JT, Huang JH, et al. Formation of enterovirus-like particle aggregates by recombinant baculoviruses co-expressing P1 and 3CD in insect cells. *Biotechnol Lett* 2003;25:919-925.

99. Ku Z, Ye X, Huang X, et al. Neutralizing antibodies induced by recombinant virus-like particles of enterovirus 71 genotype C4 inhibit infection at pre- and post-attachment steps. *PLoS One* 2013;8:e57601.

100. Cao Y, Sun P, Fu Y, et al. Formation of virus-like particles from O-type foot-and-mouth disease virus in insect cells using codon-optimized synthetic genes. *Biotechnol Lett* 2010;32:1223-1229.

101. Wetz K, Kucinski T. Influence of different ionic and pH environments on structural alterations of poliovirus and their possible relation to virus uncoating. *J Gen Virol* 1991;72 (Pt 10):2541-2544.

102.Sun D, Chen S, Cheng A, et al. Roles of the Picornaviral 3C Proteinase in the Viral Life Cycle and Host Cells. *Viruses* 2016;8:82.

103.Ypma-Wong MF, Filman DJ, Hogle JM, et al. Structural domains of the poliovirus polyprotein are major determinants for proteolytic cleavage at Gln-Gly pairs. *J Biol Chem* 1988;263:17846-17856.

104.Arnold E, Luo M, Vriend G, et al. Implications of the picornavirus capsid structure for polyprotein processing. *Proc Natl Acad Sci U S A* 1987;84:21-25.

105.Ansardi DC, Porter DC, Morrow CD. Coinfection with recombinant vaccinia viruses expressing poliovirus P1 and P3 proteins results in polyprotein processing and formation of empty capsid structures
J Virol 1991;65:2088-2092.

106.Ko YJ, Choi KS, Nah JJ, et al. Noninfectious virus-like particle antigen for detection of swine vesicular disease virus antibodies in pigs by enzyme-linked immunosorbent assay. *Clin Diagn Lab Immunol* 2005;12:922-929.

107.Porta C, Xu X, Loureiro S, et al. Efficient production of foot-and-mouth disease virus empty capsids in insect cells following down regulation of 3C protease activity. *J Virol Methods* 2013;187:406-412.

108.Rombaut B, Foriers A, Boeye A. In vitro assembly of poliovirus 14 S subunits: identification of the assembly promoting activity of infected cell extracts. *Virology* 1991;180:781-787.

109.Goodwin S, Tuthill TJ, Arias A, et al. Foot-and-mouth disease virus assembly: processing of recombinant capsid precursor by exogenous protease induces self-assembly of pentamers in vitro in a myristoylation-dependent manner. *J Virol* 2009;83:11275-11282.

110.Zlotnick A, Stray SJ. How does your virus grow? Understanding and interfering with virus assembly. *Trends Biotechnol* 2003;21:536-542.

111.Polacek C, Gullberg M, Li J, et al. Low levels of foot-and-mouth disease virus 3C protease expression are required to achieve optimal capsid protein expression and processing in mammalian cells. *J Gen Virol* 2013;94:1249-1258.

112.Chung CY, Chen CY, Lin SY, et al. Enterovirus 71 virus-like particle vaccine: improved production conditions for enhanced yield. *Vaccine* 2010;28:6951-6957.

113.Roelvink PW, van Meer MM, de Kort CA, et al. Dissimilar expression of Autographa californica multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. *J Gen Virol* 1992;73 (Pt 6):1481-1489.